# Dynamic adjustment of photosynthetic pigment composition in leaves

**Kumulative Dissertation**

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

**Kim Gabriele Beisel**

aus Karlsruhe

Oktober 2010

aus dem Institut für Chemie und Dynamik der Geosphäre 3:
Phytosphäre (ICG-3), Forschungszentrum Jülich GmbH

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# Zusammenfassung

Pflanzen haben vielfältige Kurz- und Langzeitstrategien entwickelt, um ihre photosynthetischen Membranen an unterschiedliche Lichtverhältnisse anzupassen. Das Einfangen von Lichtenergie bei gleichzeitigem Schutz vor derselbigen, unter sich ändernden Strahlungsumgebungen, ist für die Pflanze überlebenswichtig und muss in den Blättern sorgfältig reguliert und ausbalanciert werden. Einen wichtigen regulatorischen Mechanismus bilden bei diesem Prozess die Anpassung und Instandhaltung der Zusammensetzung von photosynthetischen Pigmenten.

Generelle Adaptations- und Akklimatisierungsmuster der Pigmentzusammensetzung in den Blättern bei Schatten und Sonne wurden in einer Studie mit phylogenetisch diversen Pflanzen aus tropischen Wäldern in einer natürlichen Umgebung untersucht. Spezies, die an niedrige Strahlungsintensitäten adaptiert oder akklimatisiert waren, neigten dazu relativ mehr in das Einfangen von Lichtenergie zu investieren, was sich in einer hohen massebezogenen Akkumulation von Chlorophyll (Chl) und Carotinoiden, vor allem Neoxanthin (N) und Lutein (L), manifestierte. Die Akklimatisierung an starke Sonneneinstrahlung resultierte in einem erhöhten Gehalt an photoprotektiven Pigmenten des Xanthophyll-Zyklus, das heißt Violaxanthin (V) + Antheraxanthin (A) + Zeaxanthin (Z), sowie β-Carotin (β-C), bezogen auf die Blattfläche und den Chl-Gehalt. In den meisten der untersuchten Tropenspezies wurde α-Carotin (α-C) nachgewiesen, aber das Verhältnis von α-C zu β-C in den Sonnenblättern war stets klein. Eine durchgängige Präferenz von β-C an Stelle von α-C bei starken Strahlungen lässt auf nachteilige Eigenschaften von α-C unter hohen Lichtbedingungen schließen.

Der dynamische Umsatz (turnover) von Pigmenten, in Verbindung mit der stationären (steady-state) Pigmentzusammensetzung und der Photosystem II (PSII) Effizienz, wurde in voll entwickelten Blättern der Modellpflanze Arabidopsis (*Arabidopsis thaliana*) unter Langzeit-Niedriglicht, Langzeit-Hochlicht und Kurzeit-Hochlichtstress in einer Klimakammer untersucht. Um den dynamischen Umsatz von photosynthetischen Pigmenten zu erforschen wurde eine Methode zur *in vivo* $^{14}CO_2$ Markierung ($^{14}CO_2$ pulse-chase Applikation) von Arabidopsis-Blättern sowie ein Protokoll zur Radio-HPLC Pigmentanalyse entwickelt. Die $^{14}CO_2$ Markierungsexperimente offenbarten einen kontinuierlichen Umsatz von β-C und

Chl *a* in voll entwickelten Blättern, sogar unter niedrigen Lichtverhältnissen. Kurzzeitige Hochlicht-Stressapplikation hatte keinen Einfluss auf den Umsatz von β-C und Chl *a*, führte aber zu einem signifikanten Anstieg des V+A+Z- und L-Gehaltes innerhalb weniger Stunden. Dennoch war in diesen Xanthophyllen keine Radiomarkierung nachweisbar, was unterschiedliche Ursprünge von Molekülvorstufen für den kontinuierlichen Umsatz von β-C und der stressinduzierten Biosynthese von Xanthophyllen in Chloroplasten vermuten lässt. Der Umsatz von Carotinen war signifikant erhöht, wenn ein Großteil des β-C durch α-C im Kern-Komplex von PSII und PSI durch eine Mutation im *lut5*-Gen ausgetauscht worden war, was in Übereinstimmung mit den geringen Anteilen an α-C in Sonnenblättern von tropischen Waldpflanzen ist.

Des Weiteren wurden Interaktionen zwischen dem dynamischen Umsatz von β-C und Chl *a* mittels Inhibitoren der D1 Protein- (Lincomycin) und Carotinoid-Biosynthese (Norflurazon) getestet. Die Behandlung mit Lincomycin führte zu einer gravierenden Abnahme der Markierung von Chl *a* zusammen mit einer eher moderaten Abnahme der Markierung von β-C, was auf eine enge Kopplung des Umsatzes des D1 Proteins mit dem Umsatz von Chl *a*, aber nicht mit dem Umsatz von β-C schließen lässt. Diese Hypothese ist in Einklang mit dem kleineren β-C-Umsatz und dem größeren Chl *a*-Umsatz, die in Pflanzen beobachtet wurden, die an Hochlicht-Bedingungen akklimatisiert waren. Von solchen Pflanzen ist bekannt, dass sie einen hochregulierten D1 Protein-Umsatz und PSII Reparaturzyklus haben. Eine Teilhemmung der Carotinoid-Biosynthese durch Norflurazon hat nicht nur die lockere Kopplung zwischen dem β-C- und Chl *a*-Umsatz bestätigt, sondern auch die Fähigkeit von Arabidopsis-Blättern aufgedeckt, den Stoffwechselfluss entlang des Carotinoid-Biosyntheseweges zügig (innerhalb einiger Minuten) hochzuregulieren, wenn ein Mangel an β-C vorliegt.

Die Ergebnisse, die in dieser Arbeit erzielt und diskutiert worden sind, betonen die hoch dynamische Regulation der Zusammensetzung von photosynthetischen Pigmenten in voll entwickelten Blättern unter verschiedenen Lichtbedingungen.

## Summary

Plants have evolved a number of short- and long-term strategies to acclimate and adapt their photosynthetic membranes to distinct irradiances. Fine regulation and balancing of light harvesting and photoprotection in leaves is essential for the survival of plants in changing light environments. In this respect, adjustment and maintenance of photosynthetic pigment composition represent an important regulatory mechanism.

A general picture of sun-shade adaptation or acclimation patterns of leaf pigment composition was obtained from a survey including phylogenetically diverse tropical forest plants growing under natural environments. Species adapted or acclimated to low irradiance tended to invest relatively more in light harvesting, as manifested by high mass-based accumulation of chlorophyll (Chl) and carotenoids, especially neoxanthin (N) and lutein (L). High-light acclimation increased the contents of photoprotective xanthophyll-cycle pigments, i.e. violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z), as well as β-carotene (β-C) on a leaf area and Chl basis. Most tropical forest species contained α-carotene (α-C), but the α-C to β-C ratio was always low in sun leaves. Universal preference of β-C over α-C under strong irradiance suggests penalties of α-C under high light.

Dynamic pigment turnover, in conjunction with the steady-state pigment composition and the photosystem II (PSII) efficiency, was investigated in mature leaves of a model plant Arabidopsis (*Arabidopsis thaliana*) under long-term low-light, long-term high-light and short-term high-light stress applied in a climate chamber. A method and a protocol for *in vivo* $^{14}CO_2$ pulse-chase labeling with Arabidopsis leaves and radio-HPLC pigment analysis were developed to study turnover of photosynthetic pigments. The $^{14}CO_2$ pulse-chase labeling experiments revealed continuous turnover of β-C and Chl *a* in mature leaves, even under low irradiance. Short-term high-light stress did not alter β-C or Chl *a* turnover but induced a significant increase of the V+A+Z and L contents within several hours. However, no radio-label was detected in these xanthophylls, suggesting separate precursor pools for continuous β-C turnover and stress-induced xanthophyll biosynthesis in chloroplasts. Consistent with the low abundance of α-C in sun leaves of tropical forest plants, turnover of carotenes was significantly increased when a substantial part of the β-C pool was replaced by α-C in the core complexes of PSII and PSI due to mutation to the *lut5* gene.

Interactions between β-C and Chl *a* turnover were further examined by using inhibitors of D1 protein (lincomcyin) and carotenoid synthesis (norflurazon). Treatment with lincomycin resulted in a striking decrease of Chl *a* labeling concomitant with a rather moderate decrease of β-C labeling, indicating a close coupling of D1 protein turnover with Chl *a* turnover, but not with β-C turnover. This is coherent with the lower β-C turnover and higher Chl *a* turnover found in high-light acclimated plants, in which D1 turnover and PSII repair cycle are known to be upregulated. Partial inhibition of carotenoid biosynthesis by norflurazon not only confirmed a loose coupling between turnover of β-C and Chl *a*, but also revealed the ability of Arabidopsis leaves to quickly (within minutes) upregulate metabolic flux down the carotenoid biosynthetic pathway when there is short supply of β-C.

The results obtained and discussed in this thesis underscore the highly dynamic regulation of photosynthetic pigment composition in mature leaves under changing light environments.

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# 1. Introduction

## 1.1 The Photosynthetic Apparatus and its Pigments

The overall process by which plants use light energy to synthesize organic compounds is called photosynthesis. In addition to providing biomass to the ecosystem photosynthesis produces, as a by-product, $O_2$ required for respiratory activity by almost all organisms on earth. Photosynthesis includes a complex series of reactions that involve light absorption, energy conversion, electron transfer and a multistep enzymatic pathway that converts $CO_2$ and water into carbohydrates.

In eukaryotes all steps of photosynthesis take place in chloroplasts which contain an internal membrane system, known as thylakoid membranes. Thylakoid membranes contain several multi-subunit protein complexes, of which two, photosystem I (PSI) and photosystem II (PSII), bind photosynthetic pigments, i.e. chlorophylls and carotenoids. These pigments are major players in the light-dependent reactions of photosynthesis: Some accomplish photon absorption and subsequent transfer of excitation energy to the photosystem reaction centers while others protect the photosystems from excess light and photodamage.

The PSII consists of over 20 distinct protein subunits; some are involved in harvesting of light energy and regulation of energy flow while others play a role in oxidation of water to $O_2$. The core complex of PSII is composed of proteins which specifically bind chlorophyll *a* (Chl *a*) and β-carotene (β-C), including the D1 and the D2 polypeptide and the core antenna proteins CP47 and CP43 (Bassi et al., 1993; Loll et al., 2005). The PSII core complex assembles as a dimer in the stacked regions (grana) of the thylakoid membranes (Andersson and Anderson, 1980). The outer major light-harvesting antenna of PSII (LHCII) forms a protein trimer which binds Chl *a*, Chl *b* and the xanthophylls lutein (L), neoxanthin (N) and violaxanthin (V) under non-stressful conditions (Peter and Thornber, 1991; Liu et al., 2004). Besides the peripheral LHCII, PSII contains additionally three monomeric minor antenna complexes, CP29, CP26 and CP24, which also bind Chl *a*, Chl *b* and xanthophylls (Bassi et al., 1993).

The PSI is smaller than the PSII and can be mainly found in the stroma region of the thylakoid membranes (Andersson and Anderson, 1980). The PSI core complex exists as trimer, in which each monomer is composed of 12 protein subunits which build, besides the reaction centre, a large internal antenna system with numerous Chl *a* and β-C molecules (Amunts et

al., 2010). The outer light harvesting antenna of PSI (LHCI) bound to one side of each PSI core unit consists of four, monomeric proteins containing Chl *a*, Chl *b*, β-C, L and V but only little N (Ballottari et al., 2004; Morosinotto et al., 2005; Amunts et al., 2010). PSI receives $e^-$ from the PSII via plastoquinone, cytochrome $b_6f$ complex and plastocyanin and transfers it to ferredoxin on the stromal side of the thylakoid membrane. From ferredoxin, $e^-$ can be either transferred to $NADP^+$ to produce NADPH (linear $e^-$ transport), or brought back to plastoquinone without NADPH production (cyclic $e^-$ transport). In both linear and cyclic $e^-$ transport pathways, a $[H^+]$ gradient (ΔpH) is built up across the thylakoid membrane by coupled transport of $e^-$ and $H^+$. The ΔpH and the resulting voltage gradient across the thylakoid membrane (proton-motive-force) are used by ATP synthase for production of ATP.

Together, PSII and PSI convert solar energy into chemical energy (ATP) and provide NADPH for reduction of $CO_2$ to organic compounds. Photosynthetic pigments are essential components (functional as well as structural) bound to these large protein-complexes. Table 1 summarizes the pigment composition found in PSII and PSI.

**Table 1 Pigment composition of PSI and PSII proteins** (molecules per polypeptide). Adapted from Yamamoto and Bassi (1996) and modified according to Jordan et al. (2001)[‡], Ballottari et al. (2004)†, Liu et al. (2004)[#], Loll et al. (2005)*, Morosinotto et al. (2005)[+] and Amunts et al. (2010)[§].

| | Chl *a* | Chl *b* | β-C | L | N | V |
|---|---|---|---|---|---|---|
| PSI core | 93-96[‡,§] | - | 22-26[‡,†,+] | - | - | - |
| LHCI | 63-65[§,†] | 15-17[§,†] | 13 (?) | 8[+,§] | - | 3[+,§] |
| PSII core | 35* | - | 11* | - | - | - |
| LHCII | 8[#] | 6[#] | - | 2 | 1 | 1[#] |
| D1/D2 dimer | 6 | - | 2* | - | - | - |
| CP47 | 16* | - | 5* | 0.4 (?) | - | - |
| CP43 | 13* | - | 4* | 1 (?) | - | - |
| CP29 | 6 | 2 | 0.1 | 1.3 | 0.5 | 1 |
| CP26 | 6 | 3 | 0.1 | 1.3 | 0.4 | 0.5 |
| CP24 | 3 | 2 | 0.2 | 1.2 | - | 0.5 |

## 1.2 Biosynthesis of Photosynthetic Pigments

Carotenoids and chlorophylls are large chromophore molecules that have vital functions (light harvesting and photoprotection) in chloroplasts. The biosynthesis of these pigments, partly sharing the same precursors, requires a highly regulated and concerted activity of many enzymes. The main steps of the respective pathways will be described in the following sections.

### 1.2.1 Carotenoids

Carotenoids comprise a large $C_{40}$ isoprenoid family. Nevertheless, in chloroplasts of vascular plants the composition of carotenoids is highly conserved. The isoprenoid precursors are generally synthesized via the deoxyxylulose-5-phosphate pathway (DOXP) in plastids (Lichtenthaler, 1999). Carotenoids involved in photosynthesis (photosynthetic carotenoids) are $C_{40}$ tetraterpenoids derived from phytoene (Phy). It is the first committed step of carotenoid biosynthesis to produce Phy by the condensation of two molecules of geranylgeranyl diphosphate (GGDP) by the enzyme Phy synthase (PSY) (Figure 1). In plants, Phy is formed as *cis*-isomer which is subsequently converted to all-*trans* lycopene (Lyc) by two desaturase enzymes, Phy desaturase (PDS) and ζ-carotene desaturase (ZDS) (Sandmann, 2009), and an isomerase (CRTISO) (Park et al., 2002). In green/mature leaves neither the colorless Phy nor the reddish Lyc accumulates in large amounts.

Cyclization of Lyc gives rise to two distinct branches of the biosynthetic pathway with orange/yellowish carotenoids: β- and ε-cyclization of linear Lyc leads to formation of α-carotene (α-C) and its derivatives, having one β- and one ε-ring (β,ε-branch), while β-cyclization on both ends of Lyc leads to formation of β-C and its derivatives with two β-rings (β,β-branch). The relative activity of ε- and β-cyclase enzymes determines the proportion of carotenoids that are channeled through the two branches of the pathway (Cunningham et al., 1996).

Hydroxylation in the ε- and β-ring moieties of α-C by ε- and β-hydroxylase (OHase), respectively, produces L, the most abundant xanthophyll in plant chloroplasts, and hydroxylation in both β-rings of β-C by β-OHase, different from the one responsible for α-C hydroxylation, forms the xanthophyll zeaxanthin (Z) (Kim et al. 2009). In some plant taxa (not in *Arabidopsis thaliana*) the β-ring of L can be epoxidized to lutein epoxide (Lx) (García-

Plazaola et al., 2007; Matsubara et al., 2007) whereas epoxidation in the two β-rings of Z gives rise to antheraxanthin (A) and V in all plant species. The successive formation of A and V, and probably also the production of Lx, is accomplished by the enzyme zeaxanthin epoxidase (ZE) (Siefermann and Yamamoto, 1975; Marin et al., 1996). Finally, V can be converted to neoxanthin (N) by N synthase, the last carotenoid product of the β,β-branch.

Under excess light or other environmental stress conditions part of the V pool is deepoxidized back to A and Z by the enzyme V deepoxidase (VDE) to enhance photoprotection (compare section 1.3.1) (Yamamoto et al., 1962; Bugos and Yamamoto, 1996). This light-dependent interconversion between V, A and Z by deepoxidation and epoxidation reactions is known as the xanthophyll cycle (Yamamoto et al., 1999; Jahns et al., 2009). In species containing Lx, a similar light-dependent interconversion between Lx and L can be observed, which is referred to as the Lx cycle (Bungard et al., 1999). The Lx cycle operates in parallel with the xanthophyll cycle and is probably catalyzed by the same enzymes, VDE and ZE (García-Plazaola et al., 2007). Upon high-light exposure Lx is quickly deepoxidized to L, as is V to A and Z; in contrast, epoxidation of L back to Lx is usually much slower than that of V (Matsubara et al., 2008).

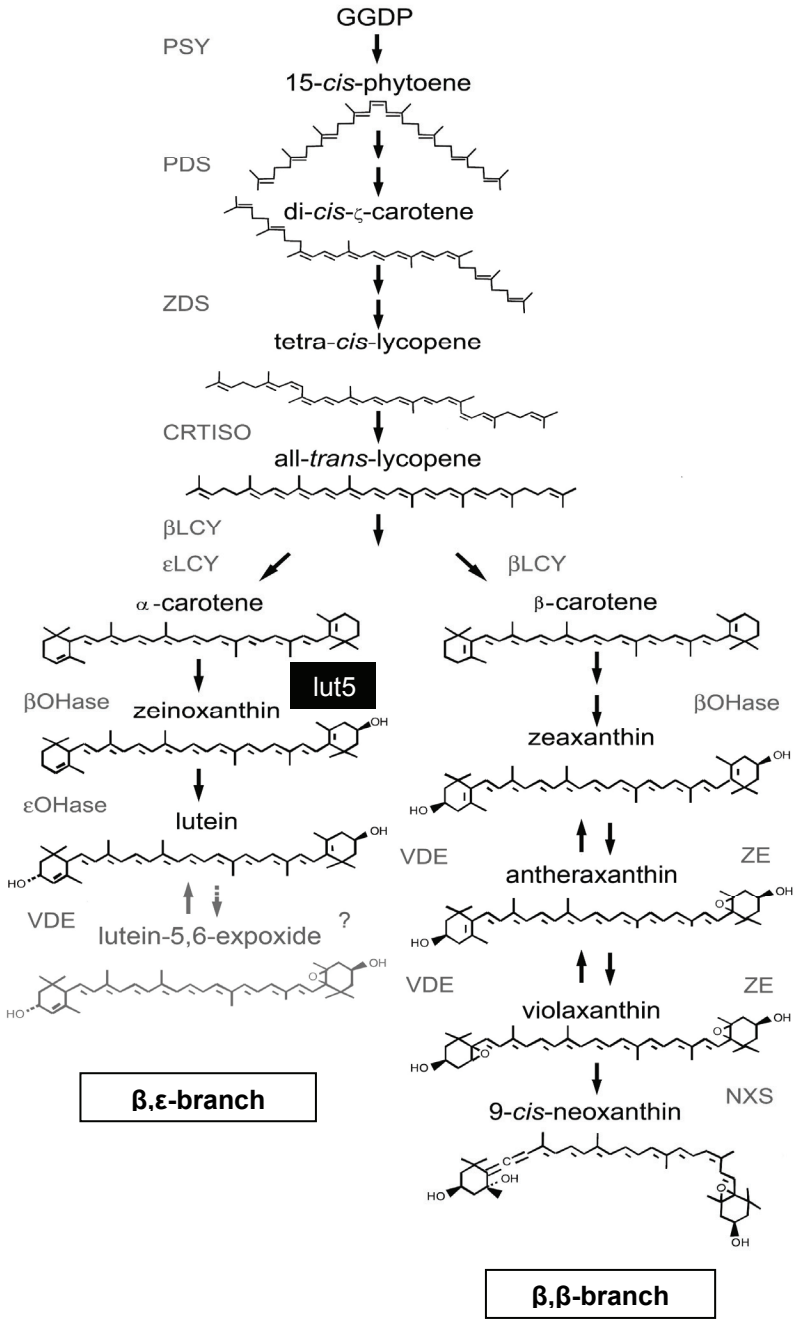


**Figure 1 Carotenoid biosynthetic pathway in plants** (scheme adapted from DellaPenna and Pogson, 2006) The abbreviation for the biosynthetic enzymes is given next to each step and the *Arabidopsis* mutation *lut5* used in this dissertation is shown as a black square. β-LCY, lycopene β-cyclase; β-OHase, β-hydroxylase; CRTISO, carotenoid isomerase; ε-LCY, lycopene ε-cyclase; ε-OHase, ε-hydroxylase; GGDP, geranylgeranyl diphosphate; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin deepoxidase; ZDS, ζ-carotene desaturase; ZE zeaxanthin epoxidase. Carotenoids having one β- and one ε-ring account for the β,ε-branch of the pathway; carotenoids with two β-rings constitute the β,β-branch.

### 1.2.2 Chlorophylls

Chlorophyll (Chl) consists of two moieties, chlorophyllide and phytol, which are formed in the pathways of tetrapyrrole and isoprenoid biosynthesis, respectively. The tetrapyrrolic branch, located in plastids, yields δ-aminolevulinic acid from glutamic acid (Figure 2). Eight δ-aminolevulinic acid molecules form the ring structure of protoporphyrin IX. Further modification of the ring system to produce chlorophyll include the insertion of $Mg^{2+}$ by magnesium chelatase and the attachment of a phytol chain by chlorophyll synthase (Tanaka and Tanaka, 2006). The isoprenoid precursors leading to the formation of the phytol chain from GGDP are also derived from the plastidial DOXP pathway, as is the case for carotenoid biosynthesis (1.2.1) (Lichtenthaler, 1999).

Chl *a* and Chl *b* differ in their substituent at the C7 position of the ring structure; a methyl group is present in the former, whereas a formyl group is present in the latter. It still has to be verified *in vivo* whether Chl *b* is synthesized directly from Chl *a* or rather from chlorophyllide *b* by the enzyme chlorophyll *a* oxygenase (von Wettstein et al., 1995; Oster et al., 2000). However, Chl *b* can be converted back to Chl *a* in the so-called chlorophyll cycle. This reaction is important for Chl *b* degradation, especially during adjustment of photosystem antenna size, resulting in changes of Chl *a* to Chl *b* ratio (Chl *a*/*b*), in response to different light regimes (discussion section 3.2) (Rüdiger, 2002).

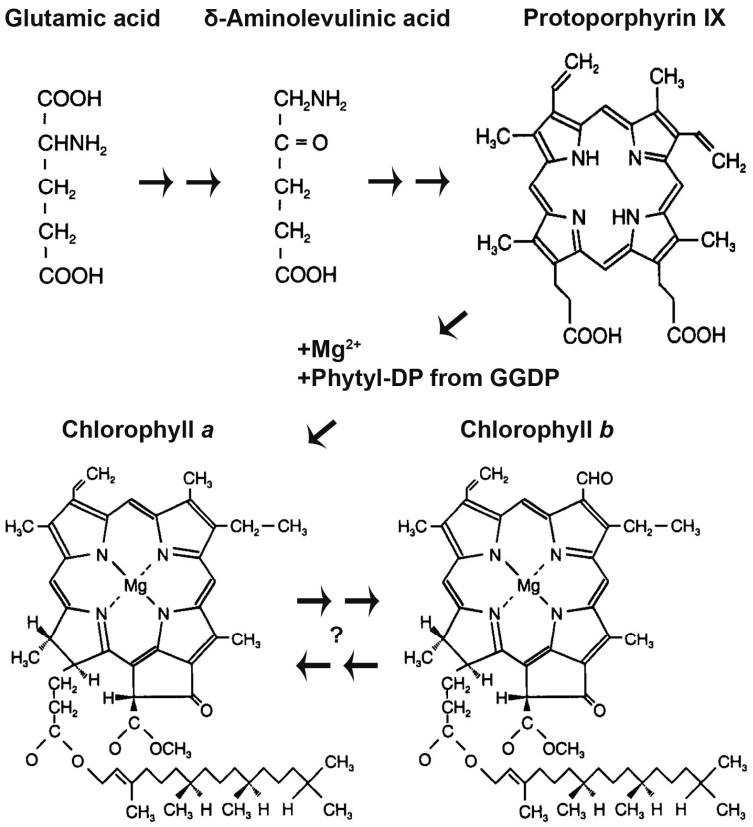


**Figure 2 Chlorophyll biosynthetic pathway in plants** (simplified scheme adapted from von Wettstein et al., 1995 and Tanaka and Tanaka, 2006). The question mark indicates reactions that are still under debate. GGDP, geranylgeranyl diphosphate; Phytyl-DP, phytyl diphosphate.

## 1.3 Photoprotection

Light is the essential energy source for photosynthesis, but light can also be harmful to the photosynthetic apparatus when the energy absorbed by pigments does not get utilized for photochemical reactions. Plants have therefore evolved various strategies to protect themselves from excess light (Demmig-Adams and Adams, 1992b; Niyogi, 1999). Three mechanisms involving photosynthetic pigments will be discussed in the following sections.

### 1.3.1 Thermal Dissipation

Photoprotection can occur by dissipation of absorbed light energy in the pigment-protein complexes. One rapid and effective mechanism is the diversion of energy away from the PSII reaction centers in form of thermal (heat) dissipation (Niyogi, 2000), as determined by measurement of so-called nonphotochemical quenching (NPQ) of Chl *a* fluorescence (compare section 1.3.2). The rapidly (within seconds) inducible and reversible component of NPQ (qE) is dependent on the trans-thylakoidal [$H^+$] gradient (ΔpH). The qE mechanism can therefore serve as a quick feedback-regulated safety valve for photosynthesis under fluctuating light intensities (Külheim et al., 2002) because it is directly controlled by the magnitude of ΔpH determined by the balance between the photosynthetic electron transport and the activity of ATP synthase. The lowering of the lumen pH activates VDE that converts V into Z as part of the xanthophyll cycle (section 1.2.1.) (Yamamoto et al., 1999; Jahns et al., 2009). In addition, a low luminal pH causes protonation and subsequent conformational change of the PsbS protein of PSII (Li et al., 2004). Both protonated PsbS and Z seem to play a prominent role in the qE mechanism. However, it is still under debate where exactly the quenching process takes place (minor or major antenna of PSII) and whether Z participates in a direct (Ahn et al., 2008) or indirect manner (Horton et al., 2008).

The sustained form of thermal dissipation is called qI. This slowly relaxing component of NPQ is often associated with photoinhibitory processes in the PSII reaction centers. Under severe photooxidative stress, ZE becomes inactivated to result in retention of Z (Reinhold et al., 2008) even in the absence of ΔpH (Verhoeven et al., 1998). This sustained retention of Z is also considered as a component of qI (Jahns and Miehe, 1996; Verhoeven et al., 1996). Further aspects of photoinhibition will be discussed in section 1.4.

### 1.3.2 Fluorescence Emission

Light energy can be either utilized for photosynthesis (section 1.1) or dissipated as heat (NPQ, section 1.3.1), but it can also be re-emitted from Chl *a* molecules in form of photons. This mechanism, referred to as chlorophyll fluorescence, can dissipate only 1 to 2% of the total light absorbed. Hence, fluorescence emission is not relevant with photoprotection. However, measuring Chl *a* fluorescence is a powerful tool for plant biologists to estimate the efficiency of PSII (Maxwell and Johnson, 2000): Changes in the quantum yield of PSII indicate an altered efficiency of photochemistry and/or heat dissipation since these three processes (fluorescence, photochemistry and dissipation) compete with each other. The maximal quantum yield of PSII is termed Fv/Fm, where Fm is the maximal fluorescence in the dark-adapted state, Fv=Fm-Fo, and Fo the minimal fluorescence in the dark-adapted state (van Kooten and Snel, 1990). The Fv/Fm values are close to 0.84 in healthy leaves (Björkman and Demmig, 1987). A decrease of Fv/Fm points to an increase of qI-quenching (section 1.3.1).

### 1.3.3 Defense against Photooxidation

If absorbed photons are neither utilized for photochemistry nor efficiently dissipated, they can generate reactive oxygen species (ROS) that cause photooxidative damage to the photosynthetic apparatus and other components of the cell (compare section 1.4) (Mittler, 2002). Plants have therefore developed mechanisms to circumvent the formation of ROS and they have acquired a broad spectrum of antioxidant compounds which detoxify ROS, once they appear.

Carotenoids serve both functions: On one hand they prevent the formation of highly reactive singlet oxygen ($^1O_2^*$) by quenching triplet Chl ($^3Chl^*$) molecules, which arise easily through intersystem crossing from excited singlet Chl ($^1Chl^*$) (Seely, 1978). $^3Chl^*$ in turn is a relatively long-living species that can react with triplet oxygen ($^3O_2$) to form harmful $^1O_2^*$ molecules. On the other hand carotenoids can also directly quench $^1O_2^*$. The former process requires close proximity of the carotenoid molecule to $^3Chl^*$ (i.e. in the pigment-protein complexes of PSII) (Di Valentin et al., 2009) while the latter can be additionally carried out by non-protein bound (free) carotenoids in the membrane (Havaux and Niyogi, 1999; Gruszecki and Strzałka, 2005). For $^3Chl^*$ quenching the major candidate is thought to be L (Dall´Osto et al., 2006, Mozzo et al., 2008). In addition, L was also found to have antioxidative properties (Peng et al., 2006). Other carotenoids that have been proposed to act as antioxidants are non-protein

bound Z (Havaux et al., 2007), reaction centre β-C (Telfer, 2003) and N in LHCII (Dall´Osto et al., 2007).

## 1.4 Photoinhibition

Despite multiple lines of protection, frequent damage to the photosynthetic apparatus, and thus inhibition of certain photosynthetic steps, is an inevitable consequence of oxygenic photosynthesis. In particular, the PSII reaction centre is susceptible to photodamage, which leads to its inactivation. Two hypotheses, both probably happening in leaves (Oguchi et al., 2009), have been proposed to explain the process of photoinactivation: The "acceptor side mechanism" postulates that accumulation of excess energy in the reaction centers (not sufficient acceptors) results in the formation of $^{3}Chl^{*}$, and consequently, in the production of $^{1}O_{2}^{*}$ which in turn causes oxidative damage to PSII if not detoxified by antioxidants (section 1.3.3) (Vass et al., 1992). The "donor side mechanism" is an $O_2$-independent route for photoinactivation; it occurs when the rate of electron donation from water to the PSII reaction centre does not keep pace with the electron removal on the acceptor side (Hakala et al., 2005). As a consequence, electrons from surrounding molecules will be extracted to bridge the gap (Andersson and Aro, 2001).

Whatever the mechanisms of photoinhibition, damaged proteins are left behind. Particularly susceptible to photooxidation is, even at low-light intensities, the reaction centre protein D1 (section 1.1) (Keren et al., 1997). At increasing irradiance damage to D1 and hence inactivation of PSII occurs more and more frequently (Tyystjärvi and Aro, 1996). However, plants have developed a very efficient repair mechanism to rapidly replace the oxidized D1 protein with a new copy of the polypeptide to restore functioning of the PSII reaction centre (Melis, 1999). The PSII repair cycle is a complex process that involves reversible phosphorylation of damaged D1 and other PSII core proteins and changes in the oligomeric structure of PSII (Baena-González and Aro, 2002). The phosphorylation by specific protein kinases is thought to facilitate unpacking of the damaged PSII complex and its migration from grana to stroma thylakoids where D1 is dephosphorylated by a still unknown phosphatase, and subsequently, degraded by a D1-specific protease (Tikkanen et al., 2008). Replacement of damaged D1 by co-translational insertion of a new protein copy and reassembly of the PSII core subunit require the availability of assembly partners (Zhang et al., 1999) and ligation of Chl *a* (Kim et al., 1994; He and Vermass, 1998) and β-C (Trebst and

Depka, 1997). As long as plants can rapidly replace the damaged D1 protein, whereby its degradation is the rate limiting step, no visible or measurable symptom can be detected *in vivo*. Only when the rate of D1 replacement cannot keep pace with the rate of photodamage, e.g. upon irradiance increase, the overall quantum yield of PSII (Fv/Fm, section 1.3.2) declines, which is referred to as photoinhibition (Aro et al., 1993). At the same time, damaged PSII reaction centers are sites of thermal dissipation (qI) (section 1.3.1), thereby contributing to protection of functional neighbors (Krause, 1988; Öquist et al., 1992; Lee et al., 2001).

# 2. Motivation

During the last three decades a great deal of research has been done to understand the regulatory processes of photosynthetic light utilization. To date we know much about the principal structure and functioning of the photosynthetic apparatus. Nevertheless, there are still a number of open questions concerning the regulation and adjustment of photosynthesis that is essential for the survival of plants in quickly changing light environments.

An important question addressed in this thesis is the adaptation and acclimation of the photosynthetic pigment composition to achieve an optimal balance between light harvesting, dissipation of excess light energy and other photoprotective reactions in a highly variable light environment. General patterns of pigment compositions associated with low and high irradiances are well-known for many years by studies on steady-state pigment levels, but little effort has been made to elucidate dynamic processes leading to such pigment phenotypes. In this work, I explored to extend the understanding of photosynthetic pigment adjustment by investigating their dynamic synthesis and degradation (turnover). To this end, methods and protocols were developed for *in vivo* $^{14}CO_2$ pulse-chase labeling in Arabidopsis leaves and for radio-HPLC analysis of leaf pigment extracts. Combining the methods and techniques to analyze both steady-state and dynamic behavior of pigments, together with the analysis of PSII activities by Chl *a* fluorescence measurements, I sought to obtain a better insight into the mechanisms enabling short- and long-term acclimation of photosynthetic membranes to changing light intensities.

# 3. Discussion

Plants have developed several short- and long-term strategies to acclimate and adapt to distinct light environments in order to achieve an optimal cost-benefit trade-off between light harvesting and photoprotection. Some sun/shade characteristics of plants are already manifested on a genotypic level and therefore constitutively present (adaptation) while others are inducible and formed in response to, amongst others, irradiances (acclimation). Specific morphological (e.g. leaf form, thickness and surface properties) and biochemical phenotypes (e.g. composition of photosynthetic pigments and proteins) can be observed as a consequence of acclimation to shade or full sunlight (Givnish, 1988; Sheperd and Griffiths, 2006; Matsubara et al. 2009). The following sections will discuss acclimatory responses to long-term low-light (section 3.1), long-term high-light (section 3.2) and short-term high-light stress (section 3.3) with the main focus on the behavior of photosynthetic pigments.

## 3.1 Acclimation and adaptation to low light

Under low-light conditions, plants invest relatively more resource in light harvesting to promote photosynthesis. They increase the photosystem antenna size (number of LHCIIs) relative to the number of core complexes, as indicated by a decline of the Chl *a* / Chl *b* ratio (Anderson and Andersson, 1988; Beisel et al. 2010, legend to Fig. 2). This regulation of antenna size is restricted to PSII, whereas PSI-LHCI stoichiometry remains constant (Ballottari et al., 2007).

An optimization of the photosynthetic quantum yield, especially under low-light conditions with occasional increases in irradiance, can be also achieved by a short-term acclimation process called state transitions (qT). When PSII becomes overexcited relative to PSI the absorbed light energy can be quickly (within minutes) redistributed by migration of a mobile pool of LHCIIs from PSII in the grana to PSI in the stroma thylakoids, and *vice versa* in the case of PSI overexcitation (Haldrup et al., 2001; Allen, 2003). The migration process is induced by reversible phosphorylation of LHCIIs by a thylakoid-bound kinase (Bonardi et al., 2005; Rochaix, 2007) which allows dissociation of LHCII from PSII. The responsible kinase is regulated via the redox state of the photosynthetic electron transport chain, especially by the plastoquinone pool (section 1.1) (Haldrup et al., 2001; Allen, 2003). Thus, whenever one of the two photosystems is preferentially excited, the redox state of the plastoquinone pool

triggers re-distribution of excitation energy between PSII and PSI to correct the imbalance and establish a new balance. It has been proposed recently that state transitions also play a role in regulation of linear and cyclic electron transport. Under preferential excitation of PSII in Chlamydomonas, a PSI-LHCI-LHCII complex, derived from state transition, was found to switch electron flux from the linear to the cyclic mode (section 1.1) (Iwai et al., 2010). Cycling of electrons around PSI, involving ferredoxin and the cytochrome $b_6f$ complex, can increase the $[H^+]$ gradient across the thylakoid membrane (ΔpH) without reduction of $NADP^+$ to NADPH, when relatively more ATP production and/or qE induction is needed (Kramer et al., 2004).

In our large survey with phylogenetically diverse tropical plants (Matsubara et al., 2009) a clear and general trend to increase the chlorophyll and carotenoid content was observed in shade leaves (Matsubara et al., 2009; Fig. 2A and B). The pigment data were expressed on a dry mass basis, given by the structural parameter "LMA" (leaf mass per area) (Wright et al., 2004), which emphasizes the economic investment in light-harvesting pigments of leaves grown under low-light conditions. In particular, there was a significant increase of the xanthophylls N and L (or Lx+L for species able to accumulate Lx) in shade leaves (Matsubara et al., 2009; Fig. 3C), probably accompanying the accumulation of LHCII to improve light harvesting; L is bound to L1 and L2 site of LHCII, and N in N1 site of LHCII (Formaggio et al., 2001; Liu et al., 2004). Lx (>5 mmol mol $Chl^{-1}$) was found in only 20% of the species examined in the survey, but across a broad range of rather unrelated plant taxa (Matsubara et al., 2009; Table 2). The ability to accumulate relatively high amounts of Lx has probably evolved independently among different plant lineages (García-Plazaola et al., 2004) and high amounts of Lx have been associated with light harvesting in shade leaves of mostly tropical plants (Matsubara et al., 2005, 2007, 2008).

The survey of tropical plants revealed also a significant increase of α-C as adaptive and/or acclimatory responses to low-light environments (Matsubara et al., 2009; Fig. 3). Accumulation of α-C has been found in shade leaves of a wide range of plants in many previous studies (e.g. Demmig-Adams and Adams, 1992a) and thus a possible function of α-C in improving light harvesting has been proposed by Krause et al. (2001). α-C probably replaces β-C (and *vice versa*) in the core complex of PSII and in the core and antenna of PSI (Matsubara et al., 2007); the stoichiometric relation between the α-C and β-C content in our

data set is consistent with their complementary binding to the same binding sites (Matsubara et al., 2009; Fig. 6). Some species had low levels of α-C in both sun and shade leaves, as seen in many crop plants whose leaves are almost free of α-C. Interestingly, accumulation of α-C seems to be a common feature in terrestrial plants that have evolved relatively early in evolution (i.e. gymnosperms and basal angiosperms) while more recent lineages (monocots and dicots) exhibit considerably lower α-C levels (Esteban et al., 2009). Hence, there is apparently an evolutionary trend to replace α-C with β-C in the core complexes of the photosystems. Also in wild-type plants of Arabidopsis, β-C is the only carotene species under natural conditions and α-C can be found only in traces. However, the absence of the *lut5* gene product, an β-OHase in the β,ε-branch (Figure 1), leads to the accumulation of α-C to the level comparable with that of β-C in the wild-type (Kim and DellaPenna, 2006; Fiore et al., 2006; Beisel et al., 2010; Fig. 5B and 2E, respectively). Our $^{14}CO_2$ pulse-chase labeling experiments with the *lut5* mutant under short-term high-light conditions (1000 µmol photons $m^{-2}$ $s^{-1}$ for up to 10 h) revealed strikingly high incorporation of $^{14}C$ in α-C (Beisel et al., 2010; Fig. 6B), up to 60% higher than the incorporation of $^{14}C$ in β-C of the respective wild-type plants (Beisel et al., 2010; Fig. 3B). These findings point toward a general instability of α-C under high light (enhanced susceptibility to photodamage), which might be another explanation, in addition to better light harvesting (Krause et al., 2001), why α-C is mainly found in shade leaves in nature.

Overall, there is a clear tendency in shade leaves to invest more in β,ε- rather than in β,β-carotenoids (Matsubara et al., 2009; Fig. 4). This pattern is mainly attributable to the elevated α-C content (at the expense of the β-C content) in leaves that have been grown in low-light conditions (Matsubara et al., 2009; Fig. 5). A possible mechanism which regulates this acclimation process could be the relative activities of the lycopene ε- and β-cyclases at the branching point in the carotenoid biosynthetic pathway (Cunningham et al., 1996; Pogson and Rissler, 2000). Additionally or alternatively, the activity of β-OHases in the β,ε-branch (see section 1.2.1) may also affect the carotene composition, as exemplified by the *lut5* mutation in Arabidopsis (Kim et al., 2009).

## 3.2 Acclimation to high light

Under high irradiance, plants can develop strong sinks (photochemistry and carbon fixation) to efficiently utilize the sunlight. They invest more in structural components, such as formation of thick leaves having smaller surface to volume ratios, and accumulate large amounts of starch, both of which lead to high LMA (Matsubara et al., 2009; Fig. 1). However, growth-related morphological acclimation processes are rather slow and mostly irreversible. Relatively quick and reversible changes in response to high-light exposure include alterations in the photosynthetic pigment patterns.

A common response of leaves to high irradiance is the reduction of PSII antenna relative to core complex in order to minimize overexcitation of the photosynthetic apparatus by excess light harvesting. The relative abundance of Chl *a* and Chl *b* can be rather quickly adjusted by the operation of the chlorophyll cycle, especially by the reduction of Chl *b* to Chl *a* under strong light (section 1.2.2) (Rüdiger, 2002). Consequently, high Chl *a* / Chl *b* ratios are a predominant acclimatory symptom found in leaves under high irradiance (Anderson et al., 1988; Beisel et al., 2010; legend to Fig. 2).

Our $^{14}CO_2$ pulse-chase labeling studies revealed generally high *de novo* synthesis and degradation of Chl *a*, but not Chl *b*, in mature leaves of Arabidopsis under light (Beisel et al., 2010; Fig. 3A). The specific turnover of Chl *a* seems to be related to the PSII reaction centre repair cycle (section 1.4); it can be drastically retarded by treatment with lincomycin, an inhibitor of D1 protein synthesis (see section 1.4 and 3.3) (Beisel et al., 2010 submitted; Fig. 4A and B). Interestingly, the $^{14}C$ incorporation in Chl *a* was significantly increased in leaves that had been acclimated to high light (1000 µmol photons $m^{-2}$ $s^{-1}$) for two weeks while short-term exposure (up to 10 h) to high light did not results in any significant change in Chl *a* labeling (Beisel et al., 2010; Fig. 3A). These results indicate an efficient up-regulation of PSII repair involving Chl *a* turnover, which is a vital photoacclimatory response of leaves to long-term high-light exposure (Aro et al., 1994); in contrast, short-term high-light stress did not immediately alter the turnover of PSII and Chl *a* significantly (see section 3.3) (Russell et al., 1995; Beisel et al., 2010; Fig. 3).

Another common feature of leaves acclimated to full sun is the large pool of the xanthophyll-cycle pigments (V+A+Z) (Demmig-Adams and Adams 1992a). These pigments, in particular Z, facilitate photoprotection as essential components of the ΔpH-induced qE

mechanism (see section 1.3.1). Expressed on a Chl basis, the average V+A+Z pool in sun leaves was found to be almost twice as high as the corresponding pool in shade leaves in the survey with 86 tropical plants species (Matsubara et al., 2009; Fig. 3B). Also in the study with Arabidopsis, an increased V+A+Z pool, by a factor of three, was recorded in leaves after two weeks of acclimation to high light (1000 µmol photons $m^{-2}$ $s^{-1}$) compared to control leaves growing under 130 µmol photons $m^{-2}$ $s^{-1}$ (Beisel et al., 2010; Fig. 2G and A, respectively). Under such long-term high-light conditions, V accounted for more than half of the total V+A+Z pool while Z made up less than 20% during the entire day period; deepoxidation of the xanthophyll-cycle pigments was obviously only partial in the high-light acclimated Arabidopsis leaves. This pattern demonstrates generally large photosynthetic and photoprotective capacities of high-light acclimated plants; leaves that constantly experience high-light intensities develop the ability to efficiently utilize light for photosynthesis and growth, and consequently, only little excess energy has to be dissipated. Under strong irradiance, large amounts of V (or V+A+Z) accumulate (Demmig-Adams and Adams 1992a; García-Plazaola et al., 2002), predominantly at the V1 site in LHCII as readily available source for the formation of Z (Caffarri et al., 2001; Jahns et al., 2009) in order to quickly induce NPQ on demand. The V+A+Z pool of high-light acclimated plants is dominated by V molecules which become accessible to VDE after quick release from LHCII into the lipid phase, and thus readily converted to A and Z under illumination, as opposed to non-convertible pool of V (presumably bound at the L2 site of the antenna proteins) which remains epoxidized even at the maximal levels of deepoxidation (Färber and Jahns, 1998; Wehner et al., 2006).

Notably, a large V+A+Z pool seems to exclude natural co-occurrence of large amounts of α-C (Matsubara et al., 2009; Fig. 7). In line with this finding, β-C can be found as the predominant, or often the only carotene species in leaves that are acclimated to full sunlight (Matsubara et al., 2009; Fig. 6). As mentioned in the previous section (3.1), β-C in the core complex of PSII seems to be less susceptible to photodamage than α-C in the same place (Beisel et al., 2010). Furthermore, β-C is proposed to have photoprotective functions (Telfer, 2003). Both properties (photostability and photoprotection) are essential for the optimal PSII activity under high-light conditions. Nevertheless, although steady-state levels of β-C typically remain unchanged under illumination (Beisel et al., 2010; Fig. 2B, E and H), our $^{14}CO_2$ pulse-chase labeling studies with Arabidopsis leaves revealed a continuous turnover of β-C, even under non-stressful light conditions (Beisel et al., 2010; Fig. 3B). This turnover of β-

C is probably partially coupled with the D1 protein turnover (Trebst and Depka, 1997) and Chl *a* turnover (see above and section 3.3) as part of the reaction centre repair cycle (Beisel et al., 2010 submitted). Surprisingly, the $^{14}C$ incorporation in β-C was found to be lower in leaves that had been acclimated to high-light conditions, conversely to the observations made for Chl *a* (see above). The $^{14}C$ level of β-C was comparable in leaves that had been subjected to short-term high-light stress and leaves that were kept under control-light conditions (Beisel et al., 2010; Fig. 3B). Obviously, strong irradiance does not lead to any short-term changes of β-C turnover, as was also found for Chl *a* (Beisel et al., 2010; Fig. 3A). Long-term acclimation to high light, on the other hand, might improve β-C recycling or, probably indirectly, stability of β-C. The photoacclimatory behavior of β-C turnover in mature leaves raises several intriguing questions and merits further investigation.

In general, leaves acclimated to high light show a clear preference for the accumulation of β,β-carotenoids over β,ε-carotenoids (Matsubara et al., 2009; Fig. 4). This acclimation response is mainly attributable to an increase of the β,β-xanthophyll content, especially the V+A+Z pool, rather than to the elevated level of β-C (Matsubara et al., 2009; Fig. 5). Hence, β-C hydroxylation probably represents an additional regulatory step after the branching point in the carotenoid biosynthetic pathway to determine the xanthophyll compositions (Davison et al., 2002).

## 3.3 Responses to high light stress

Plants which are not acclimated to high-light conditions but subjected to strong irradiance experience high-light stress. In our study with Arabidopsis plants, which were grown under 130 µmol photons $m^{-2}$ $s^{-1}$, short-term exposure to high light (1000 µmol photons $m^{-2}$ $s^{-1}$) induced quick operation of the xanthophyll cycle (Beisel et al., 2010; Fig. 2D). After approximately 1 h, all of the convertible V was deepoxidized to Z (Färber and Jahns, 1998; Wehner et al., 2006) and the V+A+Z pool increased by a factor of 2.5 after 10 h, presumably involving prompt upregulation of β-OHase gene expression (Cuttriss et al., 2007). In contrast to leaves that had been acclimated to this strong irradiance (section 3.2), Z rather than V was the predominant xanthophyll species in the V+A+Z pool (Beisel et al., 2010; Fig. 2G). Hence, leaves that are suddenly stressed by high light counteract quickly via formation and maintenance of large amounts of Z in order to enhance thermal dissipation of excess energy (section 1.3.1) and improve antioxidative capacity (section 1.3.3).

We observed an increase of L by approximately 25% after 10-h exposure to high light (Beisel et al., 2010; Fig. 2F). Notably, the increase of L was slightly larger (by 5 %) in the Arabidopsis *lut5* mutant (Beisel et al., 2010; Fig. 5C) which at the same time exhibited a less pronounced increase of the V+A+Z pool (by a factor of 2 versus 2.5 in the wild type) (Beisel et al., 2010; Fig. 5A and 2D, respectively). This may suggest similar or compensatory functions of L and Z. L is involved in light harvesting (Croce et al., 2001; Liu et al., 2004) as mentioned in section 3.1, but it also plays several critical roles in photoprotection. The importance of L as an antioxidant and effective quencher of $^{3}Chl^{*}$ has been known for a while (for details see section 1.3.3). In addition, more and more evidence is accumulating in recent years to support participation of L in thermal energy dissipation from $^{1}Chl^{*}$ (Pogson and Rissler, 2000; Bonente et al., 2008; Ruban et al., 2007). It was demonstrated, for instance, that accumulation of additional L in an Arabidopsis mutant can partly compensate the lack of A+Z with respect to qE (Li et al., 2009). Also in leaves of species having the Lx cycle, elevated levels of L are associated with upregulation of energy dissipation upon exposure to strong illumination (Matsubara et al., 2008; Förster et al., 2009). While these photoprotective roles of L are becoming established, just how L mediates energy dissipation in the antenna complexes of PSII remains unanswered and requires further investigation.

Another interesting question arises about the origin and destination of additional V+A+Z and L under short-term, stressful light conditions. The continuous increases of V+A+Z and L following the maximal deepoxidation in the xanthophyll cycle (Beisel et al., 2010; Fig. 2D and F) suggest *de novo* synthesis of these pigments. Contrary to our expectation, we could not detect any radio-labeled xanthophylls in our $^{14}CO_2$ pulse-chase labeling experiments in spite of high $^{14}C$ incorporation in β-C, the immediate precursor of V+A+Z (Beisel et al., 2010). Although we cannot rule out possible effects of technical limitations, the apparent lack of xanthophyll labeling might have physiological reasons. Since short-term high-light exposure enhances *de novo* synthesis of V+A+Z without affecting the turnover of β-C (Beisel et al., 2010; Fig. 3B), one could think of compartmentalization of carotene and xanthophyll biosynthesis, perhaps involving different carbon sources (metabolic channeling): The continuous turnover of β-C in light is directly fed by photosynthetic $CO_2$ fixation while the stress-induced accumulation of xanthophylls derives carbon supply from precursors that are not directly linked to the Calvin cycle.

A possible precursor pool for additional V+A+Z could be β-C molecules that are already present (i.e. prior to the pulse-labeling with $^{14}CO_2$) and released from photoinactivated PSII during the repair cycle, as has been proposed for Chlamydomonas by Depka et al. (1998). Although possible, this cannot be the only mechanism for xanthophyll accumulation in response to high-light stress in mature Arabidopsis leaves. Firstly, after some time (several PSII repair cycles), one would expect to find radio-labeled Z formed from radio-labeled β-C, but this was not the case. Secondly, the increase of L in wild-type leaves with no α-C cannot be explained by this theory. Moreover, treatment of Arabidopsis leaves with norflurazon, an inhibitor of carotenoid biosynthesis at the step of Phy desaturation, resulted in a significant decrease of the β-C content concomitant with repression of the V+A+Z and L increase during short-term high-light stress (Beisel et al., 2010 submitted; Fig. 3 H and I). Thus, β-C derived from PSII core complexes cannot be the only source of additional V+A+Z and L under high-light stress in Arabidopsis leaves. Interestingly, relatively high levels of $^{14}$C-labeled β-C were found in norflurazon-treated leaves along with remarkably high accumulation of radio-labeled Phy (Beisel et al., 2010 submitted; Fig. 4C to F). The observed increase of $^{14}$C-labeled β-C plus Phy (by approximately 50%) suggests strong and rapid upregulation of the carbon flux down the carotenoid biosynthetic pathway in response to the norflurazon treatment (Beisel et al., 2010 submitted; Fig. 4G and H). This observation points once more to a sensitive regulation of carotenoid biosynthesis, presumably via metabolic feedback reactions on an enzyme activity and/or at the gene transcription level (Cazzonelli and Pogson, 2010). However, quick upregulation of the pathway in response to norflurazon treatment could obviously neither provide sufficient amount of β-C to maintain the steady-state level nor allow an increase of V+A+Z and L under high-light stress (Beisel et al., 2010 submitted; Fig. 3 H and I).

There are several assumptions with respect to the localization of extra xanthophylls under high-light stress: Additional Z and L may (1) replace protein-bound V in the antenna complexes (Jahns et al., 2009), (2) occupy empty xanthophyll binding sites (i.e. L2 or V1) (Morosinotto et al., 2003), (3) accumulate at the protein-lipid interface of antenna complexes, or (4) remain free in the lipid phase of the thylakoid membrane (Dall´Osto et al., 2010). Depending on the localization, these Z and L molecules could fulfill distinct photoprotective functions, such as energy dissipation (1.3.1) and defense against photooxidation (1.3.3).

Concomitantly with the activation of the xanthophyll cycle and the synthesis of additional Z and L, a rapid decrease followed by a gradual decrease in the maximal PSII efficiency (Fv/Fm, section 1.3.2) was observed in Arabidopsis leaves exposed to high-light stress for up to 10 h (Beisel et al., 2010; Fig. 1). The strongest reduction in PSII quantum efficiency was found within the first 30 min of the experiment, reflecting slowly reversible NPQ in these dark-adapted leaves (section 1.3.1) with increasing amounts of Z (Nilkens et al., 2010). Further decrease of the Fv/Fm value could be attributed to increasing photoinhibition (section 1.4) due to accumulation of photoinactivated PSII with damaged D1 protein. Additionally or alternatively, Z, which is synthesized after maximal deepoxidation of V, might contribute also to the slow phase of Fv/Fm decline (Nilkens et al., 2010). When the same experiment was performed in the presence of lincomycin, an inhibitor of D1 protein synthesis, Fv/Fm decreased much more rapidly and strongly during the high-light treatment, reaching almost zero by 3 h (Beisel et al., 2010 submitted; Fig. 1B), despite the operation of the xanthophyll cycle and synthesis of additional Z (Beisel et al., 2010 submitted; Fig. 3E). Even under control-light conditions the maximal PSII efficiency was impaired in lincomycin-treated leaves by 25% after 6 h, while it stayed at the maximum level in water-treated leaves under the same conditions (Beisel et al., 2010 submitted; Fig. 1B and 1A, respectively). These results underline the importance of the PSII repair cycle (section 1.4) in order to keep high photosynthetic efficiency (Tyystjärvi and Aro, 1996).

As mentioned above, also Chl *a* and β-C molecules are likely involved in the PSII turnover processes (Beisel et al., 2010). However, only the turnover of Chl *a* seems to be regulated in tight coordination with the turnover of D1 protein, since treatment with lincomycin resulted in a significant reduction of Chl *a* labeling but only slightly reduced levels of $^{14}$C-β-C (Beisel et al., 2010 submitted; Fig. 4A to D). It was shown that D1 stability during the turnover process is strongly dependent on the presence of Chl *a* (Kim et al., 1994; He and Vermaas, 1998). On the other hand, treatment of Arabidopsis leaves with norflurazon (see above) led to a moderate reduction of β-C labeling without affecting the levels of $^{14}$C-Chl *a* and the maximal PSII efficiency (Beisel et al., 2010 submitted; Fig. 4A to D and Fig. 1C). Although inhibition by norflurazon was only partial in our experiments (judged by relatively high labeling of β-C), these results support the notion that the turnover of β-C is not closely coupled with Chl *a* and D1 protein turnover. This somewhat distinct regulation of β-C turnover presumably reflects requirement of β-C molecules at other sites in the thylakoid membrane and for the

synthesis of downstream xanthophylls in response to certain stress signals. Yet, it should be noted that long-term application of norflurazon during plant cultivation results in bleaching of leaves (e.g. Dalla Vecchia et al., 2001) or loss of D1 protein and PSII activity in Chlamydomonas under high-light stress (Trebst and Depka, 1997). Hence, synthesis of β-C is essential for D1 protein assembly in PSII during the repair cycle, and photo-bleaching of β-C may trigger D1 protein degradation (Trebst and Depka, 1997). Whatever the mechanisms, our data suggest different regulatory responses to high-light stress for β-C, Chl *a* and D1 protein turnover (Beisel et al., 2010 submitted). Interaction between dynamic turnover of cofactors in photosynthetic pigment-protein complexes is worth studying further in detail.

One more question that has arisen from this study is the fate of Chl *a* and β-C under non-stressful light conditions. Our labeling experiments revealed high and continuous incorporation of $^{14}$C in Chl *a* and β-C even under control-light conditions (Beisel et al., 2010; Fig. 3A and B), which is similar to the behavior known for D1 protein, i.e. showing rapid turnover even under low rates of excitation (Keren et al., 1997). One may ask whether Chl *a* and β-C in the PSII reaction centre undergo degradation to the same rate as D1 protein. The steady-state levels of Chl *a* and β-C usually do not increase over time (Beisel et al., 2010 submitted; Fig. 2A and C) despite continuous synthesis, suggesting degradation and/or partial recycling of the pigment molecules after release from photoinactivated PSII. It has been shown for *Synechocystis* that both, chlorophyllide and phytol moieties derived from chlorophyll can be reutilized for chlorophyll synthesis (Vavilin and Vermaas, 2007). This recycling mechanism, or even complete rescue of non-damaged Chl *a* molecules, could explain the gradual increase in the $^{14}$C-Chl *a* level observed in Arabidopsis leaves throughout the 6-h chase (Beisel et al., 2010; Fig. 3A). A different observation was made for β-C: The amount of $^{14}$C-β-C always reached a plateau within 0.5-1 h (Beisel et al., 2010; Fig. 3B), which suggests a fate for β-C different from recycling. For green leaves not much is known about carotenoid degradation and catabolism. In addition to chemical photooxidation, carotenoids can be degraded enzymatically by carotenoid cleavage dioxygenases (CCD; Auldrige et al., 2006), leading to, amongst others, synthesis of abscisic acid (ABA). ABA is a plant hormone important for seed development and germination, but it also plays a predominant role in leaves during drought and osmotic stress (Leung and Giraudat, 1998). However, it is unlikely that high levels of carotenoids are continuously converted into ABA under non-stressful conditions in green leaves. Moreover, ABA is not directly derived from β-C but from cleavage

of V or N (Nambara and Marion-Poll, 2005). Other carotenoid cleavage products include volatile norisoprenoids that contribute to flavor and/or aroma of fruits and flowers (Auldrige et al., 2006) and strigolactones that are secreted by roots as signaling molecule for germination of mycorrhizal fungi spores (Akiyama et al., 2005; Matusova et al.; 2005). It is not known, however, if such degradation pathways start at β-C (or α-C) also in leaves. Further research is needed to gain better insights into carotenoid degradation in green tissue to explain the phenomenon of continuous β-C turnover in photosynthesizing, non-senescent leaves.

# 4. Synopsis and Outlook

This dissertation sought to gain better insights into the mechanisms enabling short- and long-term acclimation of photosynthetic membranes to variable light environments, with the main focus set on the behavior of photosynthetic pigments. Several different methods, including HPLC, $^{14}CO_2$ pulse-chase labeling, Radio-HPLC, Chl *a* fluorescence analysis and use of Arabidopsis mutants and inhibitors, were applied to investigate steady-state and dynamic behavior of pigments in conjunction with the PSII activities under different irradiances.

Plants adapted or acclimated to low-light conditions exhibited a low Chl *a* / Chl *b* ratio (large light harvesting antennas) and increased levels of the light-harvesting pigments N and L (Matsubara et al., 2009). The majority of the tropical plant taxa included in the survey contained α-C in leaves, and the levels of α-C increased in shaded environments at the expense of β-C (Matsubara et al., 2009). This could be explained by better light harvesting of α-C in low light and/or instability of α-C in high light compared with β-C; the latter was indicated by the elevated $^{14}C$ incorporation into carotenes in *lut5* mutants (accumulating α-C in addition to β-C) in comparison with wild-type (Beisel et al., 2010). Experiments including measurements of pigment turnover and energy transfer (from carotenes to Chl) in plants that naturally contain α-C in leaves could corroborate these hypotheses about the functions of α-C and β-C in higher-plant photosynthesis.

The characteristic features found for leaves acclimated to high irradiance were a high Chl *a* / Chl *b* ratio (small light harvesting antennas), a large V+A+Z pool (high photoprotection capacity), and a large carotene pool made exclusively or largely of β-C (Matsubara et al., 2009). Higher turnover of Chl *a* observed in high-light acclimated plants (Beisel et al., 2010) suggests a correlation between Chl *a* and D1 protein turnover during the PSII repair cycle, which is upregulated during long-term exposure to strong irradiance (Aro et al., 1994). A tight coupling of Chl *a* and D1 protein turnover was also confirmed by applying the inhibitor of D1 protein synthesis (lincomycin), which resulted in severe impairment of the maximal PSII efficiency on one hand, and a rapid and significant reduction of Chl *a* synthesis on the other hand (Beisel et al., 2010 submitted). Co-regulation between Chl *a* and D1 protein turnover could be further scrutinized by using inhibitors of chlorophyll biosynthesis,

analyzing D1 protein turnover concomitant with pigment turnover, or studying mutants that exhibit an impaired PSII repair cycle.

While also present in the PSII reaction center, β-C behaved differently from Chl *a* and D1 in high-light acclimated plants, implying a somewhat different regulation of carotenoid synthesis (Beisel et al., 2010). The apparent downregulation of β-C turnover in leaves acclimated to strong irradiance invites investigations on carotenogenic enzyme expression and/or activity assays during long-term high-light acclimation. Such experiments could answer the question if high-light acclimation of β-C turnover is controlled by upstream (synthesis) or downstream (degradation) reactions in the biosynthetic pathway. Furthermore, detailed analysis of radiolabeled β-C and Chl *a* based on mass spectrometry would be of great value to quantify their turnover. This may allow estimation of the rates of synthesis, degradation and recycling.

The present study also revealed quick upregulation of metabolic flux down the carotenoid biosynthetic pathway in Arabidopsis leaves treated with the herbicide (PDS inhibitor) norflurazon; the upregulation was already detected at the end of the 0.5-h $^{14}CO_2$ pulse period, demonstrating that this is an initial response to declining β-C supply (Beisel et al., 2010 submitted). This initial upregulation was followed by a second slower upregulation (~6 h) under high-light stress. These observations suggest a sensitive and rapid metabolic feedback regulation of carotenoid accumulation in leaves. The molecular nature of these regulatory mechanisms seems to be an exciting research area in photosynthesis.

Another interesting question remains as to the source of additional V+A+Z and L that accumulated within several hours under high-light stress in Arabidopsis leaves. If they are neither directly derived from the Calvin cycle nor from β-C released from damaged PSII reaction centers during the repair cycle (Beisel et al., 2010), a different precursor pool must be postulated for stress-induced xanthophyll biosynthesis. Further efforts are needed to unravel this mystery, including the identification of precursor pools and the way the pathways utilizing these pools are separately controlled in chloroplasts.

Finally, a big question mark remains with regard to the fate of β-C which is continuously synthesized, even under non-stressful light conditions, while staying at a constant steady-state level (Beisel et al., 2010). High rates of chemical photooxidation and hydroxylation to Z seem unlikely in the absence of stress. So far, little is known about enzymatic cleavage

products of β-C in green, mature leaves. As the method for *in vivo* radiolabeling of β-C in leaves has been established in this work, downstream (as well as upstream) analysis can take advantage of this tracer technique.

Further work focusing on steps upstream and downstream of the carotenoid biosynthetic pathway would be important to get a more complete picture of the dynamic adjustment of carotenoid pigments in green leaves. The methods developed and the results obtained in this thesis lay useful and versatile foundations for future explorations deeper into the dynamic nature of photosynthetic membranes under changing environments.

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# 6. Publications of this dissertation

**In chronological order:**

**Matsubara S, Krause GH, Aranda J, Virgo A, Beisel KG, Jahns P, Winter K** (2009) Sun-shade patterns of leaf carotenoid composition in 86 species of neotropical forest plants. Funct Plant Biol **36:** 20-36

**Beisel KG, Jahnke S, Hofmann D, Köppchen S, Schurr U, Matsubara S** (2010) Continuous turnover of carotenes and chlorophyll *a* in mature leaves of Arabidopsis revealed by $^{14}CO_2$ pulse-chase labeling. Plant Physiol **152**: 2188-2199

**Beisel KG, Schurr U, Matsubara S** (submitted 2010) Altered turnover of β-carotene and chlorophyll *a* in Arabidopsis leaves treated with lincomycin or norflurazon. Plant Physiol

## 6.1 Sun-Shade Patterns of Leaf Carotenoid Composition in 86 Species of Neotropical Forest Plants (Matsubara et al., 2009)

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# Sun-shade patterns of leaf carotenoid composition in 86 species of neotropical forest plants

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**Abstract.** A survey of photosynthetic pigments, including 86 species from 64 families, was conducted for leaves of neotropical vascular plants to study sun-shade patterns in carotenoid biosynthesis and occurrence of α-carotene (α-Car) and lutein epoxide (Lx). Under low light, leaves invested less in structural components and more in light harvesting, as manifested by low leaf dry mass per area (LMA) and enhanced mass-based accumulation of chlorophyll (Chl) and carotenoids, especially lutein and neoxanthin. Under high irradiance, LMA was greater and β-carotene (β-Car) and violaxanthin-cycle pool increased on a leaf area or Chl basis. The majority of plants contained α-Car in leaves, but the α- to β-Car ratio was always low in the sun, suggesting preference for β-Car in strong light. Shade and sun leaves had similar β,ε-carotenoid contents per unit Chl, whereas sun leaves had more β,β-carotenoids than shade leaves. Accumulation of Lx in leaves was found to be widely distributed among taxa: >5 mmol mol $Chl^{-1}$ in 20% of all species examined and >10 mmol mol $Chl^{-1}$ in 10% of woody species. In *Virola elongata* (Benth.) Warb, having substantial Lx in both leaf types, the Lx cycle was operating on a daily basis although Lx restoration in the dark was delayed compared with violaxanthin restoration.

**Additional keywords:** carotene, carotenoid biosynthesis, chlorophyll, leaf dry mass, *Virola*, xanthophyll cycle.

## Introduction

Carotenoid composition in chloroplasts is highly conserved among vascular plants. Typically, leaves contain β-carotene (β-Car), lutein (L), violaxanthin (V) and neoxanthin (N) (Goodwin 1965; Young 1993). Part of V is converted to antheraxanthin (A) and zeaxanthin (Z) under excess light or other environmental stress conditions (Yamamoto *et al*. 1962). Together with chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*), most of the carotenoids are bound in the pigment-protein complexes of PSI and PSII, with the majority of xanthophylls (L, N, V, A and Z) found in the light-harvesting antenna complexes and β-Car in the core complexes (Yamamoto and Bassi 1996). Light-dependent de-epoxidation and epoxidation between V, A and Z, called xanthophyll cycle (or V cycle), play a central role in the protection of photosynthetic membranes against photooxidative damage (Demmig *et al*. 1987; Müller *et al*. 2001). The pool size of the V-cycle pigments (VAZ) exhibits pronounced sun-shade responses, being larger in sun than in shade leaves, which highlights the significance of the V cycle for photoprotection under strong light (Demmig-Adams and Adams 1992; Demmig-Adams 1998).

In higher plants, carotenoids are synthesised only in plastids (for reviews on carotenoid biosynthetic pathways in plants, see Cunningham and Gannt 1998; Hirschberg 2001; DellaPenna and Pogson 2006). Cyclisation of lycopene, the last common precursor of the photosynthetic carotenoids mentioned above, gives rise to two branches of the biosynthetic pathway: β-cyclisation on both ends of lycopene leads to formation of β-Car and its derivatives, having two β-rings (β,β-carotenoids), and β-cyclisation and ε-cyclisation at each end of lycopene leads to formation of α-carotene (α-Car) and its derivatives, having one β- and one ε-ring (β,ε-carotenoids). Hydroxylation in the ring moieties of β- and α-Car produces Z and L, respectively, and subsequent stepwise epoxidation in the two 3-hydroxy-β-rings of Z leads to formation of A and V. Thus, the V cycle operates in the downstream of β-Car, reversing the flux in the β,β-branch of the biosynthetic pathway by light-induced de-epoxidation of V to A and Z. Finally, isomerisation and modification in an epoxy-β-ring of V give rise to N.

Unlike the β,β-branch that synthesises five carotenoids (β-Car, Z, A, V and N) commonly found in chloroplasts of higher plants, L is the only ubiquitous pigment produced in the β,ε-branch and accumulates in substantial amounts. Yet, accumulation of α-Car, the first product in the β,ε-branch and the precursor of L, has been reported for leaves of many different species, especially shade-tolerant or shade-grown plants (Thayer and Björkman 1990; Demmig-Adams and Adams 1992; Siefermann-Harms 1994; Demmig-Adams 1998). Furthermore,

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in the downstream of the β,ε-branch, epoxidation in the 3-hydroxy-β-ring of L can take place in leaves and other green tissues of certain taxa to result in substantial accumulation of lutein epoxide (Lx) and one-step xanthophyll cycling between L and Lx (Lx cycle) (García-Plazaola *et al*. 2007). Similar to the occurrence of α-Car, large Lx amounts have been found predominantly in shade leaves. Thus, possible functions of α-Car and Lx in light harvesting have been proposed for species adapted (or tolerant of) to shade environments (Krause *et al*. 2001; Matsubara *et al*. 2005, 2007, 2008). At the same time, enhanced accumulation of these pigments related to shade acclimation or adaptation may suggest general upregulation of carotenoid biosynthesis in the β,ε-branch, or changes in the balance between the fluxes down the β,ε- and β,β-branch.

Dense vegetations in tropical forests create contrasting light environments, ranging from deep shade on the forest floor to full sunlight in outer canopy. If α-Car and Lx contribute to efficient light harvesting in low-light environments, and if this confers an evolutionary advantage, leaves growing on the forest floor or inside the canopy would contain higher levels of α-Car and Lx than outer-canopy leaves in a wide variety of tropical forest species. Indeed, it has been documented that shade leaves of some neotropical forest plants have large amounts of α-Car and/or Lx (Königer *et al*. 1995; Krause *et al*. 2001, 2003, 2004; Matsubara *et al*. 2008), although the number of species examined in these studies was not large enough to see whether these pigments occur widely in many different species under shaded environments. Therefore, we conducted a pigment survey on leaf carotenoid composition in tropical forest plants, including 86 species from 64 families growing in different types of tropical forests in Panama. Leaf samples were collected from understorey plants (shade) as well as inner and outer canopy (shade and sun, respectively) to compare levels of Chl as well as different β,ε- and β,β-carotenoids. In addition, leaf dry mass per area (LMA), an important leaf structural parameter (Reich *et al*. 1997; Wright *et al*. 2004), was determined to explore a possible relationship between LMA and carotenoid contents. Furthermore, operation of the Lx cycle was investigated in *Virola elongata* (Benth.) Warb, a tree species that showed high Lx amounts in both shade and sun leaves in the survey.

## Materials and methods

### *Plant material and collection sites*

Species were chosen randomly for leaf collection in different habitats in central Panama (9°N, 79–80°W) during the late wet season in November–December 2006 and 2007. Outer-canopy sun leaves were collected besides shade leaves when available and/or accessible. For two species (*Elaeis oleifera* (Kunth) Cortés and *Psychotria poeppigiana* Müll. Arg.), 'sun leaves' were taken from forest gaps and probably were not fully sun-acclimated. In total, 86 species from 64 plant families (two fern families, one gymnosperm family and 61 angiosperm families) were included in the survey. The angiosperm families considered represent ~30% of the monocotyledon and 28% of the dicotyledon families reported for Panama (Correa *et al*. 2004).

All species included in the study, their botanical naming authorities, life forms and collection sites are listed in Table 1. For nomenclature and authorities, the database of the Missouri Botanical Garden (www.mobot.org, accessed 21 November 2008) was used. Tropical forest sites for leaf collection were (1) Soberania National Park and Gamboa area, seasonally dry lowland forest on the Caribbean side of the dividing range; (2) Metropolitan Nature Park, near Panama City, seasonally dry semi-deciduous lowland forest on the Pacific side of the dividing range; (3) Barro Colorado Island Nature Monument, Lake Gatún, Panama Canal area, moist, seasonally dry lowland forest; (4) San Lorenzo National Park, moist primary lowland forest on the Caribbean slope; (5) Galeta Island Nature Reserve, mangrove swamp at Caribbean coast; (6) Altos de Campana National Park, montane cloud forest (~800 m above sea level); (7) Chagres National Park, Cerro Jefe, montane cloud forest (~900 m above sea level). At sites (2) and (4), canopy leaves of mature trees and leaves of lianas were accessible from construction cranes. Climate data for some of the study sites can be obtained at http://striweb.si.edu/esp/physical_monitoring/index_phy_mon.htm (accessed 21 November 2008).

### *Diel course of Lx and V cycles in* Virola elongata *leaves*

Diel variations in Lx and V were studied *in situ* in January 2007 (early dry season) on a mature tree of *Virola elongata* (Benth.) Warb. at collection site (4); the same tree was chosen for the pigment survey in the rainy season. Shade leaves were selected from the north-east side underneath the dense crown of the tree, where PAR remained <100 μmol photons $m^{-2}$ $s^{-1}$. Leaf discs (0.95 $cm^2$) were sampled between early morning and midday and in the following morning. Because of strong wind, discs had to be taken from different leaves at each sampling time. The discs were frozen immediately in liquid nitrogen for pigment assay. At around midday, five sun leaves were collected and dark-adapted at ambient temperature. After 2 and 4.5 h of dark adaptation, sample discs were taken from these leaves to study restoration kinetics of Lx and V.

The ratio of maximal variable to maximal total of Chl fluorescence intensity, $F_v/F_m$, indicating the potential efficiency of PSII, was recorded with a MINI-PAM fluorometer (Walz, Effeltrich, Germany) after 10 min dark adaptation, as described by Krause *et al*. (2006). Photosynthetically active radiation was measured with a LI-190SA sensor connected to a LI-189 data logger (Li-Cor, Lincoln, NE, USA).

### *Analysis of photosynthetic pigments*

For the pigment survey, whole leaves or, depending on leaf size, sections of leaf blades were detached and kept moist and dark at ambient temperature for at least 4 h before sampling of discs (0.95 $cm^2$). The samples were frozen in liquid nitrogen, stored at −70 to −80°C and transported to Germany on dry ice for HPLC analysis of photosynthetic pigments. The major part of leaf samples was collected in November 2006 and analysed at the Institut für Phytosphäre, Forschungszentrum Jülich; a minor part was collected in November 2007 and analysed at the Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf. Pigments were extracted with acetone and quantified by HPLC using the method described by Matsubara *et al*. (2005) and by Krause *et al*. (2003) based on the method by Färber *et al*. (1997).

**Table 1. List of species, author names, life forms and collection sites of tropical plants studied**
Collection sites are numbered as follows: (1) Soberania National Park and Gamboa area; (2) Metropolitan Nature Park, near Panama City; (3) Barro Colorado Island Nature Monument, Lake Gatún; (4) San Lorenzo National Park; (5) Galeta Island Nature Reserve; (6) Altos de Campana National Park; (7) Chagres National Park, Cerro Jefe

| Species and botanical naming authorities | Life form | Site |
|---|---|---|
| *Aechmea magdalenae* (André) André ex Baker | Herb | 1 |
| *Albizia guachapele* (Kunth) Dugand | Tree | 2 |
| *Amphitecna latifolia* (Mill.) A.H.Gentry | Tree | 5 |
| *Anacardium excelsum* (Kunth) Skeels | Tree | 2 |
| *Anthurium cerrocampanense* Croat | Herb | 6 |
| *Aphelandra campanensis* Durkee | Herb | 6 |
| *Avicennia germinans* (L.) L. | Tree/mangrove | 5 |
| *Brassia caudata* (L.) Lindl. | Herb/epiphyte | 6 |
| *Bursera simaruba* (L.) Sarg. | Tree | 1 |
| *Calathea lutea* Schult. | Herb | 1 |
| *Carapa guianensis* Aubl. | Tree | 4 |
| *Carludovica palmata* Ruiz & Pav. | Herb | 1 |
| *Cassipourea elliptica* (Sw.) Poir. | Tree | 6 |
| *Cavendishia stenophylla* A.C.Sm. | Shrub/epiphyte | 6 |
| *Cecropia peltata* L. | Tree | 2 |
| *Cespedezia macrophylla* Seem. | Tree | 4 |
| *Chlorophora tinctoria* (L.) Gaudich. ex Benth. | Tree | 2 |
| *Chrysobalanus icaco* L. | Shrub | 5 |
| *Chusquea simpliciflora* Munro | Herb | 1 |
| *Clusia pratensis* Seem. | Shrub | 1 |
| *Coccoloba uvifera* (L.) L. | Tree | 5 |
| *Columnea billbergiana* Beurl. | Shrub/epiphyte | 6 |
| *Combretum fruticosum* (Loefl.) Stuntz | Liana | 2 |
| *Connarus williamsii* Britton | Liana | 3 |
| *Costus villosissimus* Jacq. | Herb | 1 |
| *Cryosophila warscewiczii* (H.Wendl.) Bartlett | Tree | 6 |
| *Cyathea microdonta* (Desv.) Domin | Tree | 1 |
| *Cyclopeltis semicordata* (Sw.) J.Sm. | Herb | 1 |
| *Dalbergia monetaria* L.f. | Shrub/tree | 5 |
| *Daphnopsis americana* (Mill.) J.R.Johnst. | Shrub | 6 |
| *Dendropanax arboreus* (L.) Decne. & Planch. | Tree | 6 |
| *Dendrophthora ambigua* Kuijt | Shrub/mistletoe | 7 |
| *Desmopsis panamensis* (B.L. Rob.) Saff. | Shrub | 3 |
| *Dieffenbachia longispatha* Engl. & K. Krause | Herb | 1 |
| *Dioscorea racemosa* (Klotzsch) Uline | Liana | 6 |
| *Elaeis oleifera* (Kunth) Cortés | Tree | 1 |
| *Freziera candicans* Tul. | Tree | 7 |
| *Gouania lupuloides* (L.) Urb. | Liana | 3 |
| *Gustavia superba* (Kunth) O.Berg | Tree | 1 |
| *Hedyosmum bonplandianum* Kunth | Tree | 6 |
| *Heliconia irrasa* Lane ex R.R. Sm. | Herb | 6 |
| *Hybanthus prunifolius* (Humb. & Bonpl. ex Roem. & Schult.) Schulze-Menz | Shrub | 3 |
| *Hyeronima alchorneoides* Allemão | Tree | 1 |
| *Licania jefensis* Prance | Shrub | 7 |
| *Lindackeria laurina* C.Presl | Tree | 1 |
| *Lisianthus jefensis* A.Robyns & T.S.Elias | Shrub | 7 |
| *Luehea seemannii* Triana & Planch. | Tree | 1 |
| *Marila laxiflora* Rusby | Tree | 4 |
| *Miconia argentea* (Sw.) DC. | Tree | 1 |
| *Mikania leiostachya* Benth. | Liana | 2 |
| *Monstera dilacerata* (K.Koch & Sello) K.Koch | Liana | 6 |
| *Myrcia sylvatica* (G.Mey.) DC. | Tree | 7 |
| *Ormosia macrocalyx* Ducke | Tree | 1 |
| *Otoba novogranatensis* Mouldenske | Tree | 6 |
| *Parathesis bicolor* Lundell | Shrub | 7 |
| *Passiflora vitifolia* Kunth | Liana | 1 |

**Table 1.** (*continued*)

| Species and botanical naming authorities | Life form | Site |
|---|---|---|
| *Pera arborea* Mutis | Tree | 1 |
| *Petrea aspera* Turcz. | Liana | 3 |
| *Pharus latifolius* L. | Herb | 1 |
| *Phryganocydia corymbosa* (Vent.) Bureau ex K.Schum. | Liana | 2 |
| *Pilocosta campanensis* (Almeda & Wiffin) | Herb | 6 |
| *Piper reticulatum* L. | Shrub | 1 |
| *Pitcairnia atrorubens* (Beer) Baker | Herb | 6 |
| *Podocarpus oleifolius* D.Don ex Lamb. | Tree | 6 |
| *Psychotria poeppigiana* Műll. Arg. | Shrub | 1 |
| *Quassia amara* L. | Shrub | 3 |
| *Renealmia cernua* (Sw. ex Roem. & Schult.) J.F.Macbr. | Herb | 6 |
| *Rhizophora mangle* L. | Tree/mangrove | 5 |
| *Scleria latifolia* Sw. | Herb | 6 |
| *Serjania mexicana* (L.) Willd. | Liana | 2 |
| *Spondias mombin* L. | Tree | 2 |
| *Stigmaphyllon hypargyreum* Triana & Planch. | Liana | 2 |
| *Strychnos panamensis* Seem. | Shrub | 3 |
| *Tapirira guianensis* Aubl. | Tree | 4 |
| *Tectaria incisa* Cav. | Herb | 1 |
| *Terminalia amazonia* (J.F.Gmel.) Exell | Tree | 7 |
| *Tontelea ovalifolia* (Miers) A.C.Sm. | Liana | 4 |
| *Trichostigma octandrum* (L.) H.Walter | Liana | 2 |
| *Turnera panamensis* Urb. | Shrub | 3 |
| *Vanilla planifolia* Andrews | Liana | 1 |
| *Vantanea depleta* McPherson | Tree | 4 |
| *Virola elongata* (Benth.) Warb. | Tree | 4 |
| *Virola sebifera* Aubl. | Tree | 4 |
| *Vitis tiliifolia* Humb. & Bonpl. ex Roem. & Schult. | Liana | 2 |
| *Vochysia ferruginea* Mart. | Tree | 4 |
| *Xiphidium caeruleum* Aubl. | Herb | 6 |

### *Determination of leaf dry mass*

Additional leaf discs (0.95 $cm^2$) collected from sun and shade leaves were used to determine dry weight. These discs were also frozen in liquid nitrogen immediately after the removal and stored at –70 to −80°C before and after transportation from Panama to Germany as described above for the samples of pigment analysis. The discs were lyophilised overnight and their dry mass determined by using an analytical balance (Explorer, Ohaus, NJ). Two replicate discs were measured for each species and leaf type except for *Freziera candicans* Tul. (sun), *Myrcia sylvatica* (G.Mey.) DC. (sun and shade) and *Parathesis bicolor* Lundell (sun), for which only one leaf disc was available.

### *Statistical analysis*

Pigment data were statistically tested by using SigmaStat (SYSTAT Software GmbH, Erkrath, Germany). When datasets showed a normal distribution and similar variances between sun and shade, *t*-test (Student *t*-test) was used for analysis. When datasets failed to satisfy these conditions, a non-parametric rank sum test (Mann–Whitney Rank Sum Test) was used.

## Results

### *Pigment survey*

Total Chl contents (Chl *a*+*b*), Chl *a* to Chl *b* ratios (Chl *a*/*b*), carotenoid contents (based on Chl *a*+*b*) and ratios between α- and β-Car (α-/β-Car) in leaves of the species tested are presented in Table 2. Families of ferns, gymnosperms, monocotelydons and dicotyledons, respectively, have been ordered alphabetically. In 38 species, in which both sun and shade leaves were assayed (two species from forest gaps not considered), there was a strong tendency to higher Chl *a*/*b* ratios in sun compared with shade leaves (significant difference with $P < 0.05$ in 27 species); 29 species exhibited significantly higher VAZ in sun than in shade leaves. In 25 species, α-/β-Car was significantly higher in shade compared with sun leaves. In contrast, no α-Car was detected in shade leaves of *Scleria latiolia* (Cyperaceae), *Vanilla planifolia* (Orchidaceae) and in both leaf types of *Bursera simaruba* (Burseraceae). The values of α-/β-Car were very low in both leaf types of *Albizia guachapele* (Fabaceae) and *Lysanthus jefensis* (Gentianaceae); in five species, for which only shade leaves were tested, α-Car levels were very low (<5 mmol mol $Chl^{-1}$) or undetectable. For the family Rhizophoraceae, α-/β-Car was very high in shade leaves of one of the two species tested (*Cassipourea elliptica*, from a montane cloud forest), whereas it was extremely low in the other (*Rhizophora mangle*, from a mangrove stand).

Of the 86 species studied, >10 mmol Lx mol $Chl^{-1}$ was found in shade leaves of eight species from Arecaceae, Annonaceae, Fabaceae, Hippocrateaceae and Myristicaceae families (Table 2). In the Fabaceae species, sun leaves exhibited lower Lx levels (5–10 mmol mol $Chl^{-1}$) than shade leaves, similar to

**Table 2. Pigment contents in dark-adapted sun and shade leaves of tropical plant species**

Means ± s.e. are presented ($n = 3$, from individual leaves or leaflets) for Chl $a+b$ (μmol $m^{-2}$), Chl $a/b$ ratio (mol $mol^{-1}$) and the following carotenoids (mmol mol Chl $a+b^{-1}$): N, neoxanthin; VAZ, sum of viola-, anthera- and zeaxanthin; L, lutein; Lx, lutein epoxide; α-Car, α-carotene; β-Car, β-carotene; n.d., not detectable. Where appropriate and available, the ratio α-/β-Car (mol $mol^{-1}$) and leaf dry mass per area (LMA, g $m^{-2}$) are also given. Values of LMA are means of two samples except for those marked with an asterisk (*), for which only one sample was analysed. The LMA data are meant to be used to study general trends among many different species, as in Figs 1–3; they are not for comparisons of individual species. Data mentioned in the text are shown in bold font

| Family and species | Leaf type | Chl *a+b* | Chl *a/b* | N | VAZ | L | Lx | α-Car | β-Car | α-/β-Car | LMA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FERNS | | | | | | | | | | | |
| Cyatheaceae | | | | | | | | | | | |
| *Cyathea microdonta* | Shade | 524 ± 11 | 3.17 ± 0.05 | 34.9 ± 0.7 | 24.6 ± 0.4 | 119.3 ± 2.3 | n.d. | 27.1 ± 1.8 | 38.4 ± 0.9 | 0.70 ± 0.03 | 33.7 |
| Dryopteridaceae | | | | | | | | | | | |
| *Cyclopeltis semicordata* | Shade | 477 ± 80 | 2.99 ± 0.11 | 34.7 ± 0.2 | 23.0 ± 1.0 | 115.9 ± 1.3 | n.d. | 32.3 ± 1.6 | 23.1 ± 1.2 | 1.42 ± 0.15 | 34.7 |
| *Tectaria incisa* | Shade | 411 ± 32 | 2.53 ± 0.21 | 32.2 ± 2.8 | 24.2 ± 1.9 | 111.8 ± 6.2 | n.d. | 34.8 ± 2.0 | 27.4 ± 2.3 | 1.29 ± 0.14 | 25.3 |
| GYMNOSPERMS | | | | | | | | | | | |
| Podocarpaceae | | | | | | | | | | | |
| *Podocarpus oleifolius* | Shade | 742 ± 40 | 3.11 ± 0.08 | 36.7 ± 0.4 | 30.4 ± 1.0 | 116.3 ± 2.2 | n.d. | 47.4 ± 0.6 | 26.4 ± 0.1 | 1.80 ± 0.03 | 83.2 |
| ANGIOSPERMS-MONOCOTYLEDONS | | | | | | | | | | | |
| Araceae | | | | | | | | | | | |
| *Anthurium cerrocampanense* | Shade | 548 ± 39 | 3.21 ± 0.10 | 35.2 ± 1.0 | 21.5 ± 1.1 | 126.2 ± 4.3 | 3.7 ± 0.5 | 40.9 ± 2.9 | 21.2 ± 0.2 | 1.92 ± 0.12 | 59.5 |
| *Dieffenbachia longispatha* | Shade | 652 ± 13 | 3.48 ± 0.02 | 35.1 ± 0.5 | 24.4 ± 0.6 | 104.5 ± 3.3 | n.d. | 55.0 ± 2.0 | 20.5 ± 0.5 | 2.69 ± 0.15 | 50.0 |
| *Monstera dilacerata* | Shade | 657 ± 28 | 3.13 ± 0.09 | 40.3 ± 1.1 | 22.8 ± 2.1 | 120.4 ± 4.5 | 1.5 ± 0.3 | 37.3 ± 1.2 | 29.9 ± 0.8 | 1.24 ± 0.01 | 53.2 |
| Arecaceae | | | | | | | | | | | |
| *Cryosophila warscewiczii* | Shade | 523 ± 50 | 3.65 ± 0.17 | 28.2 ± 0.3 | 19.9 ± 1.5 | 96.9 ± 2.2 | 4.0 ± 1.5 | 53.4 ± 2.4 | 19.9 ± 0.4 | 2.68 ± 0.10 | 34.2 |
| *Elaeis oleifera* | Sun (gap) | 745 ± 25 | 3.46 ± 0.16 | 33.0 ± 1.5 | 25.9 ± 5.1 | 104.1 ± 14.7 | **12.3 ± 7.5** | 36.9 ± 5.1 | 52.8 ± 7.8 | 0.73 ± 0.23 | 77.4 |
| | Shade | 684 ± 26 | 3.32 ± 0.02 | 34.1 ± 0.5 | 16.1 ± 0.7 | 99.2 ± 1.7 | **11.0 ± 2.9** | 40.9 ± 2.7 | 45.0 ± 0.5 | 0.91 ± 0.07 | 80.3 |
| Bromeliaceae | | | | | | | | | | | |
| *Aechmea magdalenae* | Shade | 822 ± 78 | 3.32 ± 0.06 | 37.0 ± 0.8 | 28.2 ± 1.2 | 126.6 ± 4.5 | 2.4 ± 1.3 | 48.1 ± 1.4 | 27.8 ± 0.8 | 1.74 ± 0.08 | 140.0 |
| *Pitcairnia atrorubens* | Shade | 776 ± 36 | 3.08 ± 0.01 | 31.9 ± 1.9 | 33.4 ± 2.5 | 108.5 ± 2.6 | n.d. | 35.7 ± 1.9 | 30.0 ± 1.3 | 1.02 ± 0.07 | 50.5 |
| Cyclanthaceae | | | | | | | | | | | |
| *Carludovica palmata* | Shade | 592 ± 44 | 3.14 ± 0.10 | 32.5 ± 2.0 | 17.5 ± 0.4 | 117.1 ± 10.4 | n.d. | 42.5 ± 1.6 | 21.3 ± 1.4 | 2.00 ± 0.06 | 53.7 |
| Cyperaceae | | | | | | | | | | | |
| *Scleria latifolia* | Shade | 418 ± 1 | 3.26 ± 0.12 | 47.0 ± 1.2 | 29.6 ± 1.2 | 150.3 ± 2.5 | 5.0 ± 0.6 | **n.d.** | 81.1 ± 2.1 | | 32.6 |
| Dioscoreaceae | | | | | | | | | | | |
| *Dioscorea racemosa* | Shade | 462 ± 28 | 3.05 ± 0.05 | 29.0 ± 0.9 | 24.9 ± 0.1 | 100.0 ± 0.5 | n.d. | 37.2 ± 1.1 | 33.3 ± 1.8 | 1.12 ± 0.03 | 31.6 |
| Haemodoraceae | | | | | | | | | | | |
| *Xiphidium caeruleum* | Shade | 577 ± 9 | 3.21 ± 0.04 | 38.8 ± 1.2 | 26.3 ± 1.1 | 126.1 ± 3.9 | 2.4 ± 0.6 | 31.2 ± 0.5 | 33.8 ± 0.2 | 0.92 ± 0.01 | 31.6 |
| Heliconiaceae | | | | | | | | | | | |
| *Heliconia irrasa* | Shade | 359 ± 23 | 3.52 ± 0.20 | 46.1 ± 3.6 | 33.8 ± 2.7 | 141.0 ± 7.8 | 1.6 ± 0.8 | 55.0 ± 3.5 | 41.1 ± 2.8 | 1.34 ± 0.05 | 63.2 |
| Marantaceae | | | | | | | | | | | |
| *Calathea lutea* | Shade | 695 ± 73 | 3.62 ± 0.08 | 37.0 ± 0.4 | 23.6 ± 0.7 | 109.6 ± 2.5 | 8.4 ± 2.6 | 54.3 ± 1.6 | 22.5 ± 1.5 | 2.45 ± 0.22 | 48.4 |
| Orchidaceae | | | | | | | | | | | |
| *Brassia caudata* | Shade | 545 ± 15 | 2.69 ± 0.07 | 39.3 ± 1.8 | 12.6 ± 0.6 | 145.8 ± 4.4 | 7.1 ± 0.9 | 3.9 ± 1.2 | 47.7 ± 1.1 | 0.08 ± 0.02 | 44.7 |
| *Vanilla planifolia* | Sun | 309 ± 33 | 3.41 ± 0.07 | 43.7 ± 1.7 | 85.0 ± 3.9 | 249.2 ± 7.6 | 2.2 ± 1.2 | 3.1 ± 0.5 | 69.7 ± 8.2 | 0.05 ± 0.01 | 116.8 |
| | Shade | 309 ± 13 | 2.91 ± 0.11 | 45.7 ± 1.5 | 29.0 ± 2.6 | 201.3 ± 5.4 | n.d. | **n.d.** | 63.0 ± 7.9 | | 95.8 |
| Poaceae | | | | | | | | | | | |
| *Chusquea simpliciflora* | Shade | 375 ± 17 | 3.47 ± 0.04 | 36.1 ± 0.6 | 19.4 ± 0.6 | 111.5 ± 2.5 | n.d. | 46.2 ± 2.1 | 32.3 ± 2.1 | 1.45 ± 0.17 | 36.3 |
| *Pharus latifolius* | Shade | 316 ± 23 | 4.21 ± 0.47 | 21.5 ± 0.5 | 23.1 ± 1.4 | 88.6 ± 1.9 | n.d. | 23.2 ± 0.9 | 32.3 ± 1.4 | 0.72 ± 0.06 | 21.6 |
| Zingiberaceae | | | | | | | | | | | |
| *Costus villosissimus* | Shade | 192 ± 14 | 2.39 ± 0.16 | 51.6 ± 1.9 | 26.6 ± 0.8 | 174.6 ± 5.0 | 5.4 ± 0.1 | 73.1 ± 4.3 | 37.5 ± 2.0 | 1.96 ± 0.18 | 31.1 |
| *Renealmia cernua* | Shade | 351 ± 50 | 3.53 ± 0.13 | 31.6 ± 1.4 | 21.5 ± 1.9 | 103.8 ± 2.7 | n.d. | 59.0 ± 3.9 | 34.3 ± 1.7 | 1.73 ± 0.12 | 34.7 |
| ANGIOSPERMS-DICOTYLEDONS | | | | | | | | | | | |
| Acanthaceae | | | | | | | | | | | |
| *Aphelandra campanensis* | Shade | 650 ± 39 | 2.78 ± 0.01 | 37.0 ± 1.5 | 28.3 ± 0.9 | 112.0 ± 0.3 | 2.6 ± 0.3 | 28.6 ± 1.2 | 25.7 ± 1.0 | 1.11 ± 0.05 | 36.8 |
| Anacardiaceae | | | | | | | | | | | |
| *Anacardium excelsum* | Sun | 485 ± 26 | 4.04 ± 0.03 | 35.5 ± 0.4 | 52.7 ± 1.7 | 110.4 ± 2.1 | 2.6 ± 0.0 | 11.5 ± 0.9 | 68.3 ± 1.3 | 0.17 ± 0.01 | |
| | Shade | 739 ± 25 | 3.27 ± 0.07 | 38.6 ± 0.4 | 19.9 ± 0.3 | 125.7 ± 2.0 | 6.0 ± 0.6 | 30.5 ± 1.1 | 33.6 ± 2.9 | 0.93 ± 0.10 | |
| *Spondias mombin* | Sun | 475 ± 26 | 3.48 ± 0.09 | 35.8 ± 1.1 | 34.3 ± 0.4 | 124.8 ± 2.2 | 1.2 ± 0.6 | 3.2 ± 1.7 | 72.5 ± 1.6 | 0.04 ± 0.02 | 90.0 |
| | Shade | 574 ± 6 | 3.24 ± 0.06 | 37.5 ± 1.6 | 26.0 ± 1.8 | 132.9 ± 1.5 | n.d. | 15.0 ± 4.3 | 67.6 ± 2.6 | 0.27 ± 0.09 | 81.1 |
| *Tapirira guianensis* | Sun | 612 ± 11 | 3.76 ± 0.01 | 29.5 ± 0.3 | 50.8 ± 1.1 | 115.8 ± 0.7 | n.d. | 8.7 ± 0.3 | 73.0 ± 0.4 | 0.12 ± 0.00 | 104.2 |
| | Shade | 821 ± 17 | 3.08 ± 0.06 | 34.0 ± 0.5 | 28.5 ± 1.2 | 135.8 ± 3.9 | n.d. | 15.8 ± 2.8 | 53.7 ± 2.5 | 0.30 ± 0.07 | 110.0 |

**Table 2.** (*continued*)

| Family and species | Leaf type | Chl *a*+*b* | Chl *a*/*b* | N | VAZ | L | Lx | α-Car | β-Car | α-/β-Car | LMA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Annonaceae | | | | | | | | | | | |
| *Desmopsis panamensis* | Shade | 353 ± 12 | 3.37 ± 0.08 | 34.6 ± 0.8 | 19.3 ± 0.3 | 129.9 ± 1.3 | **10.0 ± 1.4** | 47.3 ± 0.1 | 26.6 ± 0.4 | 1.78 ± 0.03 | 32.6 |
| Araliaceae | | | | | | | | | | | |
| *Dendropanax arboreus* | Shade | 720 ± 15 | 2.99 ± 0.06 | 31.1 ± 1.3 | 25.1 ± 0.7 | 108.4 ± 2.8 | 1.4 ± 0.7 | 27.7 ± 2.2 | 29.8 ± 0.5 | 0.93 ± 0.06 | 44.2 |
| Asteraceae | | | | | | | | | | | |
| *Mikania leiostachya* | Sun | 548 ± 18 | 3.54 ± 0.05 | 27.6 ± 0.3 | 56.3 ± 1.0 | 110.8 ± 1.0 | n.d. | 5.7 ± 0.3 | 66.3 ± 0.6 | 0.09 ± 0.00 | 71.1 |
| | Shade | 520 ± 31 | 3.38 ± 0.03 | 30.1 ± 0.6 | 31.9 ± 0.6 | 107.4 ± 1.8 | n.d. | 9.0 ± 0.8 | 54.3 ± 0.8 | 0.17 ± 0.02 | 60.5 |
| Bignoniaceae | | | | | | | | | | | |
| *Amphitecna latifolia* | Shade | 643 ± 64 | 3.25 ± 0.03 | 35.4 ± 0.3 | 27.7 ± 0.5 | 127.4 ± 1.3 | n.d. | 31.5 ± 0.9 | 36.7 ± 1.6 | 0.86 ± 0.06 | 74.2 |
| *Phryganocydia corymbosa* | Sun | 683 ± 43 | 4.17 ± 0.08 | 26.3 ± 0.2 | 47.0 ± 2.6 | 98.7 ± 2.4 | 1.0 ± 0.5 | 13.0 ± 0.8 | 48.8 ± 2.1 | 0.27 ± 0.02 | 91.1 |
| | Shade | 502 ± 21 | 3.50 ± 0.09 | 30.7 ± 0.1 | 37.2 ± 1.9 | 113.3 ± 0.9 | 0.6 ± 0.6 | 18.4 ± 2.8 | 42.7 ± 1.9 | 0.44 ± 0.09 | 80.0 |
| Burseraceae | | | | | | | | | | | |
| *Bursera simaruba* | Sun | 372 ± 39 | 4.47 ± 0.12 | 30.9 ± 1.7 | 43.5 ± 2.4 | 108.9 ± 2.2 | 5.6 ± 0.9 | **n.d.** | 82.6 ± 0.8 | | 88.4 |
| | Shade | 405 ± 7 | 3.55 ± 0.02 | 33.4 ± 0.6 | 29.2 ± 0.9 | 107.8 ± 1.0 | 1.6 ± 0.1 | **n.d.** | 64.9 ± 0.3 | | 42.1 |
| Cecropiaceae | | | | | | | | | | | |
| *Cecropia peltata* | Sun | 529 ± 18 | 4.36 ± 0.09 | 32.9 ± 1.2 | 56.8 ± 6.3 | 89.1 ± 3.8 | 0.7 ± 0.7 | 4.8 ± 1.7 | 74.9 ± 2.3 | 0.07 ± 0.03 | 74.7 |
| | Shade | 470 ± 53 | 4.08 ± 0.11 | 33.0 ± 1.7 | 40.4 ± 2.7 | 95.1 ± 0.4 | 1.0 ± 0.5 | 12.1 ± 2.8 | 65.2 ± 3.8 | 0.19 ± 0.05 | 64.2 |
| Chloranthaceae | | | | | | | | | | | |
| *Hedyosmum bonplandianum* | Shade | 649 ± 11 | 3.00 ± 0.01 | 31.9 ± 1.6 | 21.8 ± 2.3 | 115.3 ± 6.0 | n.d. | 31.1 ± 1.1 | 25.5 ± 0.7 | 1.22 ± 0.07 | 45.3 |
| Chrysobalanaceae | | | | | | | | | | | |
| *Chrysobalanus icaco* | Shade | 526 ± 12 | 3.08 ± 0.00 | 38.5 ± 1.0 | 25.1 ± 0.6 | 150.3 ± 0.9 | 2.7 ± 0.3 | 17.7 ± 0.9 | 54.5 ± 3.2 | 0.33 ± 0.03 | 91.1 |
| *Licania jefensis* | Sun | 243 ± 11 | 5.15 ± 0.07 | 32.7 ± 1.0 | 82.7 ± 6.2 | 153.0 ± 5.3 | n.d. | 24.0 ± 1.2 | 76.9 ± 2.2 | 0.31 ± 0.02 | 140.5 |
| | Shade | 381 ± 5 | 3.92 ± 0.02 | 33.1 ± 0.3 | 33.1 ± 1.7 | 136.9 ± 3.6 | n.d. | 45.4 ± 1.7 | 44.9 ± 3.0 | 1.02 ± 0.10 | 128.9 |
| Clusiaceae | | | | | | | | | | | |
| *Clusia pratensis* | Sun | 251 ± 17 | 2.44 ± 0.11 | 45.8 ± 2.3 | 63.1 ± 8.9 | 211.7 ± 11.9 | n.d. | n.d. | 103 ± 5.5 | | |
| *Marila laxiflora* | Sun | 377 ± 9 | 3.61 ± 0.05 | 35.9 ± 1.1 | 54.7 ± 2.6 | 138.7 ± 2.4 | n.d. | 19.6 ± 1.6 | 61.4 ± 2.1 | 0.32 ± 0.03 | 115.3 |
| | Shade | 512 ± 33 | 3.32 ± 0.08 | 39.1 ± 0.8 | 31.6 ± 1.8 | 137.3 ± 4.5 | n.d. | 47.6 ± 4.3 | 41.2 ± 4.0 | 1.20 ± 0.22 | 106.3 |
| Combretaceae | | | | | | | | | | | |
| *Combretum fruticosum* | Sun | 594 ± 26 | 4.00 ± 0.11 | 33.2 ± 0.1 | 53.0 ± 1.9 | 118.2 ± 0.9 | 2.7 ± 0.2 | 14.5 ± 1.5 | 55.4 ± 0.8 | 0.26 ± 0.03 | 62.1 |
| | Shade | 619 ± 41 | 3.21 ± 0.11 | 32.6 ± 1.7 | 32.6 ± 0.4 | 102.7 ± 1.8 | 9.3 ± 0.7 | 24.6 ± 1.8 | 34.6 ± 1.0 | 0.71 ± 0.07 | 90.5 |
| *Terminalia amazonia* | Sun | 542 ± 80 | 3.57 ± 0.06 | 31.1 ± 1.3 | 37.9 ± 2.8 | 121.0 ± 3.0 | n.d. | 9.0 ± 1.1 | 59.8 ± 3.2 | 0.15 ± 0.03 | 118.4 |
| | Shade | 685 ± 54 | 2.88 ± 0.04 | 38.1 ± 1.6 | 29.6 ± 1.5 | 113.9 ± 2.9 | 1.3 ± 0.7 | 19.4 ± 1.9 | 29.2 ± 0.3 | 0.50 ± 0.05 | 95.8 |
| Connaraceae | | | | | | | | | | | |
| *Connarus williamsii* | Shade | 373 ± 12 | 3.63 ± 0.07 | 34.6 ± 0.4 | 21.7 ± 0.4 | 128.0 ± 1.6 | 1.7 ± 0.9 | 55.6 ± 1.3 | 31.3 ± 0.3 | 1.78 ± 0.02 | 46.3 |
| Ericaceae | | | | | | | | | | | |
| *Cavendishia stenophylla* | Shade | 558 ± 9 | 2.88 ± 0.02 | 31.1 ± 0.7 | 21.9 ± 0.3 | 115.3 ± 2.2 | 3.5 ± 0.3 | 0.7 ± 0.1 | 53.6 ± 1.2 | 0.01 ± 0.00 | 76.8 |
| Euphorbiaceae | | | | | | | | | | | |
| *Hyeronima alchorneoides* | Sun | 622 ± 19 | 3.36 ± 0.15 | 29.5 ± 1.2 | 33.3 ± 1.0 | 99.4 ± 4.6 | 0.4 ± 0.4 | 26.6 ± 4.0 | 44.0 ± 2.5 | 0.62 ± 0.13 | 51.6 |
| | Shade | 509 ± 38 | 2.97 ± 0.14 | 31.7 ± 1.7 | 27.7 ± 0.9 | 101.8 ± 2.1 | 3.0 ± 0.4 | 30.5 ± 0.6 | 34.8 ± 2.1 | 0.88 ± 0.04 | 43.2 |
| *Pera arborea* | Sun | 248 ± 4 | 4.58 ± 0.05 | 29.4 ± 0.2 | 26.2 ± 1.0 | 165.5 ± 4.5 | n.d. | 5.9 ± 0.5 | 95.9 ± 3.5 | 0.06 ± 0.00 | 72.1 |
| | Shade | 681 ± 17 | 3.58 ± 0.08 | 34.5 ± 0.5 | 24.3 ± 0.6 | 110.0 ± 2.5 | 1.5 ± 0.4 | 43.0 ± 0.9 | 33.6 ± 1.7 | 1.29 ± 0.07 | 53.2 |
| Fabaceae-Mimosoideae | | | | | | | | | | | |
| *Albizia guachapele* | Sun | 322 ± 14 | 5.22 ± 0.14 | 43.8 ± 1.2 | 57.8 ± 9.3 | 150.0 ± 4.6 | 6.2 ± 0.7 | 5.5 ± 0.4 | 90.8 ± 1.7 | **0.06 ± 0.00** | 60.0 |
| | Shade | 507 ± 39 | 3.79 ± 0.02 | 35.8 ± 1.3 | 26.4 ± 1.9 | 121.2 ± 8.6 | **30.9 ± 6.3** | 5.4 ± 1.3 | 81.9 ± 5.9 | **0.07 ± 0.02** | 78.9 |
| Fabaceae-Papilionoideae | | | | | | | | | | | |
| *Dalbergia monetaria* | Sun | 809 ± 75 | 3.95 ± 0.06 | 33.0 ± 0.4 | 39.3 ± 2.0 | 95.4 ± 1.6 | 5.9 ± 0.5 | 5.1 ± 0.5 | 86.1 ± 2.1 | 0.06 ± 0.01 | 103.2 |
| | Shade | 934 ± 89 | 3.16 ± 0.13 | 38.2 ± 1.6 | 21.1 ± 2.5 | 116.8 ± 5.6 | **16.3 ± 2.4** | 18.0 ± 4.4 | 58.3 ± 4.9 | 0.32 ± 0.10 | 80.3 |
| *Ormosia macrocalyx* | Sun | 637 ± 37 | 3.75 ± 0.12 | 36.8 ± 0.9 | 36.2 ± 10.2 | 150.8 ± 6.8 | 0.7 ± 0.7 | 9.9 ± 2.7 | 63.4 ± 4.1 | 0.16 ± 0.05 | 92.1 |
| | Shade | 740 ± 28 | 3.09 ± 0.04 | 38.5 ± 0.6 | 15.0 ± 0.7 | 138.6 ± 1.7 | 6.7 ± 0.6 | 20.9 ± 1.9 | 40.8 ± 1.8 | 0.52 ± 0.07 | 65.8 |
| Flacourtiaceae | | | | | | | | | | | |
| *Lindackeria laurina* | Sun | 484 ± 19 | 4.13 ± 0.09 | 38.6 ± 0.5 | 41.0 ± 2.3 | 125.6 ± 4.3 | 1.6 ± 1.6 | 15.5 ± 1.1 | 53.8 ± 0.5 | 0.29 ± 0.02 | 57.9 |
| | Shade | 575 ± 26 | 3.88 ± 0.14 | 37.2 ± 0.9 | 34.9 ± 1.3 | 119.5 ± 1.5 | 3.5 ± 0.9 | 23.0 ± 3.7 | 53.0 ± 5.8 | 0.46 ± 0.12 | 65.3 |
| Gentianaceae | | | | | | | | | | | |
| *Lisianthus jefensis* | Sun | 757 ± 59 | 3.51 ± 0.09 | 45.5 ± 1.2 | 58.3 ± 3.9 | 143.1 ± 2.1 | n.d. | 1.5 ± 0.2 | 57.4 ± 5.1 | **0.03 ± 0.00** | 105.8 |
| | Shade | 722 ± 42 | 3.35 ± 0.06 | 41.2 ± 1.5 | 20.9 ± 1.9 | 122.6 ± 1.7 | n.d. | 1.5 ± 0.2 | 48.1 ± 3.8 | **0.03 ± 0.01** | 52.1 |
| Gesneriaceae | | | | | | | | | | | |
| *Columnea billbergiana* | Shade | 439 ± 33 | 3.15 ± 0.02 | 31.4 ± 1.0 | 32.2 ± 0.2 | 105.6 ± 5.0 | n.d. | 13.1 ± 3.4 | 40.8 ± 3.1 | 0.31 ± 0.06 | 93.2 |
| Hippocrateaceae | | | | | | | | | | | |
| *Tontelea ovalifolia* | Sun | 635 ± 76 | 3.47 ± 0.19 | 38.1 ± 1.5 | 72.0 ± 4.3 | 158.2 ± 10.3 | 3.6 ± 0.5 | 17.2 ± 2.1 | 44.2 ± 3.2 | 0.39 ± 0.02 | |
| | Shade | 819 ± 41 | 3.39 ± 0.08 | 40.0 ± 1.0 | 31.5 ± 1.9 | 115.5 ± 1.6 | **11.5 ± 1.3** | 40.7 ± 2.6 | 21.4 ± 2.5 | 2.00 ± 0.39 | |
| Humiriaceae | | | | | | | | | | | |
| *Vantanea depleta* | Sun | 478 ± 24 | 4.04 ± 0.02 | 32.7 ± 0.3 | 62.1 ± 3.0 | 119.9 ± 2.7 | n.d. | 10.1 ± 1.1 | 77.5 ± 1.6 | 0.13 ± 0.02 | 112.6 |
| | Shade | 900 ± 27 | 3.03 ± 0.07 | 37.5 ± 0.5 | 25.3 ± 0.8 | 122.5 ± 1.6 | 3.3 ± 0.5 | 48.5 ± 1.0 | 29.8 ± 1.5 | 1.63 ± 0.05 | 130.5 |
| Lecythidaceae | | | | | | | | | | | |
| *Gustavia superba* | Shade | 846 ± 53 | 3.28 ± 0.03 | 39.7 ± 0.5 | 23.5 ± 0.1 | 110.6 ± 2.3 | 5.2 ± 0.4 | 53.9 ± 0.6 | 20.9 ± 0.8 | 2.59 ± 0.07 | 53.7 |

**Table 2.** (*continued*)

| Family and species | Leaf type | Chl *a*+*b* | Chl *a*/*b* | N | VAZ | L | Lx | α-Car | β-Car | α-/β-Car | LMA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Loganiaceae | | | | | | | | | | | |
| *Strychnos panamensis* | Shade | 330 ± 10 | 3.28 ± 0.03 | 26.9 ± 0.3 | 14.8 ± 0.2 | 103.2 ± 2.2 | 4.0 ± 1.2 | 36.2 ± 0.5 | 18.9 ± 1.3 | 1.93 ± 0.11 | 41.1 |
| Malpighiaceae | | | | | | | | | | | |
| *Stigmaphyllon* | Sun | 499 ± 24 | 3.85 ± 0.05 | 38.1 ± 1.5 | 52.1 ± 3.8 | 113.6 ± 4.4 | n.d. | 11.2 ± 0.4 | 66.7 ± 1.6 | 0.17 ± 0.01 | |
| *hypargyreum* | Shade | 729 ± 29 | 3.08 ± 0.08 | 45.1 ± 1.0 | 19.0 ± 1.3 | 152.7 ± 2.7 | 3.0 ± 0.2 | 40.9 ± 1.0 | 33.2 ± 1.3 | 1.24 ± 0.07 | |
| Melastomataceae | | | | | | | | | | | |
| *Miconia argentea* | Sun | 454 ± 13 | 3.45 ± 0.09 | 29.7 ± 0.8 | 33.1 ± 4.0 | 130.6 ± 14.3 | n.d. | 28.9 ± 6.8 | 47.0 ± 8.0 | 0.70 ± 0.25 | |
| | Shade | 486 ± 16 | 2.89 ± 0.10 | 34.1 ± 1.0 | 15.0 ± 0.6 | 117.7 ± 6.7 | 1.4 ± 0.4 | 54.1 ± 1.0 | 22.5 ± 1.1 | 2.42 ± 0.07 | |
| *Pilocosta campanensis* | Shade | 298 ± 6 | 3.22 ± 0.06 | 29.5 ± 1.3 | 21.9 ± 0.3 | 112.9 ± 1.5 | n.d. | 14.8 ± 0.4 | 45.0 ± 1.5 | 0.33 ± 0.02 | 28.9 |
| Meliaceae | | | | | | | | | | | |
| *Carapa guianensis* | Sun | 406 ± 19 | 4.45 ± 0.23 | 34.0 ± 5.5 | 56.5 ± 5.9 | 126.3 ± 5.3 | n.d. | 9.9 ± 0.8 | 67.8 ± 0.5 | 0.15 ± 0.01 | 102.1 |
| | Shade | 467 ± 19 | 3.83 ± 0.24 | 36.1 ± 1.0 | 33.2 ± 2.3 | 122.5 ± 1.7 | 2.5 ± 0.3 | 15.8 ± 1.3 | 54.0 ± 2.6 | 0.29 ± 0.03 | 112.1 |
| Moraceae | | | | | | | | | | | |
| *Chlorophora tinctoria* | Sun | 699 ± 30 | 4.09 ± 0.04 | 36.1 ± 0.6 | 42.5 ± 1.5 | 138.9 ± 1.1 | 2.2 ± 0.3 | 3.0 ± 0.4 | 76.8 ± 1.1 | 0.04 ± 0.01 | 82.6 |
| | Shade | 632 ± 29 | 3.61 ± 0.07 | 37.3 ± 0.3 | 24.9 ± 0.4 | 129.9 ± 4.7 | 2.5 ± 0.2 | 10.4 ± 2.7 | 54.6 ± 1.6 | 0.19 ± 0.06 | 75.3 |
| Myristicaceae | | | | | | | | | | | |
| *Otoba novogranatensis* | Sun | 426 ± 37 | 3.35 ± 0.04 | 31.5 ± 0.4 | 37.3 ± 1.5 | 102.3 ± 7.6 | **10.4 ± 0.7** | 17.6 ± 3.5 | 59.5 ± 4.0 | 0.30 ± 0.08 | 104.2 |
| | Shade | 519 ± 41 | 2.85 ± 0.07 | 34.6 ± 1.0 | 16.2 ± 1.2 | 85.3 ± 3.1 | **25.1 ± 0.9** | 46.0 ± 2.2 | 21.4 ± 1.1 | 2.15 ± 0.12 | 60.7 |
| *Virola elongata* | Sun | 565 ± 8 | 3.46 ± 0.16 | 33.4 ± 1.2 | 35.6 ± 4.8 | 94.4 ± 1.0 | **57.9 ± 2.4** | 15.6 ± 4.7 | 67.7 ± 4.9 | 0.24 ± 0.09 | 148.0 |
| | Shade | 929 ± 99 | 2.91 ± 0.06 | 39.0 ± 1.8 | 13.6 ± 2.3 | 101.3 ± 7.0 | **37.6 ± 5.8** | 45.4 ± 0.8 | 26.7 ± 2.2 | 1.71 ± 0.13 | 134.7 |
| *Virola sebifera* | Sun | 241 ± 24 | 2.33 ± 0.25 | 43.0 ± 1.7 | 59.1 ± 4.3 | 128.3 ± 5.5 | **53.4 ± 6.9** | 47.8 ± 5.1 | 93.7 ± 7.5 | 0.51 ± 0.08 | 66.6 |
| | Shade | 541 ± 46 | 2.14 ± 0.02 | 50.9 ± 0.6 | 18.8 ± 1.4 | 129.1 ± 2.9 | **46.7 ± 0.6** | 68.5 ± 2.3 | 31.9 ± 2.0 | 2.15 ± 0.07 | 63.2 |
| Myrsinaceae | | | | | | | | | | | |
| *Parathesis bicolor* | Sun | 238 ± 15 | 4.59 ± 0.13 | 33.2 ± 1.0 | 60.6 ± 4.9 | 117.8 ± 2.2 | n.d. | 7.4 ± 0.8 | 72.2 ± 1.1 | 0.10 ± 0.01 | 155.8* |
| | Shade | 455 ± 71 | 3.64 ± 0.16 | 37.5 ± 0.9 | 33.9 ± 1.0 | 120.2 ± 1.9 | n.d. | 21.6 ± 4.0 | 52.6 ± 4.3 | 0.43 ± 0.10 | 125.8 |
| Myrtaceae | | | | | | | | | | | |
| *Myrcia sylvatica* | Sun | 384 ± 14 | 3.76 ± 0.12 | 30.1 ± 0.4 | 28.8 ± 2.5 | 122.7 ± 7.5 | n.d. | 17.8 ± 1.9 | 53.3 ± 3.3 | 0.34 ± 0.06 | 132.6* |
| | Shade | 568 ± 39 | 3.16 ± 0.08 | 39.6 ± 1.0 | 31.6 ± 1.9 | 133.4 ± 5.7 | 1.6 ± 0.2 | 28.1 ± 3.5 | 39.5 ± 7.2 | 0.78 ± 0.20 | **182.1*** |
| Ochnaceae | | | | | | | | | | | |
| *Cespedezia macrophylla* | Sun | 652 ± 67 | 3.62 ± 0.04 | 35.3 ± 2.9 | 30.8 ± 2.7 | 145.3 ± 7.2 | 0.9 ± 0.9 | 33.2 ± 4.2 | 45.6 ± 1.9 | 0.73 ± 0.08 | 90.5 |
| | Shade | 553 ± 26 | 3.76 ± 0.05 | 39.0 ± 1.7 | 23.9 ± 3.7 | 129.0 ± 3.8 | 0.6 ± 0.6 | 40.8 ± 5.0 | 36.7 ± 5.9 | 1.21 ± 0.31 | 68.9 |
| Passifloraceae | | | | | | | | | | | |
| *Passiflora vitifolia* | Sun | 205 ± 12 | 4.79 ± 0.30 | 28.5 ± 0.9 | 80.9 ± 6.2 | 114.4 ± 3.6 | n.d. | 4.5 ± 0.4 | 81.0 ± 2.8 | 0.06 ± 0.01 | |
| | Shade | 373 ± 3 | 3.44 ± 0.00 | 34.4 ± 0.3 | 25.0 ± 1.1 | 117.2 ± 0.9 | n.d. | 36.2 ± 2.6 | 32.9 ± 0.7 | 1.11 ± 0.10 | |
| Phytolaccaceae | | | | | | | | | | | |
| *Trichostigma octandrum* | Sun | 551 ± 31 | 4.13 ± 0.09 | 39.4 ± 0.3 | 66.5 ± 4.1 | 136.2 ± 1.2 | n.d. | 24.5 ± 1.4 | 67.3 ± 0.6 | 0.36 ± 0.07 | 61.1 |
| | Shade | 718 ± 97 | 3.98 ± 0.11 | 33.1 ± 0.6 | 21.8 ± 0.6 | 100.0 ± 5.2 | 0.4 ± 0.4 | 37.6 ± 2.7 | 37.1 ± 1.7 | 1.02 ± 0.11 | 61.6 |
| Piperaceae | | | | | | | | | | | |
| *Piper reticulatum* | Shade | 754 ± 73 | 3.19 ± 0.08 | 37.6 ± 1.9 | 34.3 ± 0.4 | 101.4 ± 3.7 | 1.3 ± 0.0 | 47.3 ± 2.1 | 22.1 ± 0.1 | 2.14 ± 0.11 | 42.1 |
| Polygonaceae | | | | | | | | | | | |
| *Coccoloba uvifera* | Shade | 678 ± 27 | 3.24 ± 0.05 | 34.9 ± 0.5 | 32.7 ± 0.1 | 142.5 ± 1.3 | n.d. | 15.4 ± 2.2 | 47.7 ± 3.0 | 0.33 ± 0.06 | 86.3 |
| Rhamnaceae | | | | | | | | | | | |
| *Gouania lupuloides* | Shade | 214 ± 20 | 3.30 ± 0.07 | 29.2 ± 1.3 | 27.3 ± 3.5 | 126.8 ± 6.1 | n.d. | 43.7 ± 2.8 | 31.0 ± 1.5 | 1.42 ± 0.16 | 51.1 |
| Rhizophoraceae | | | | | | | | | | | |
| *Cassipourea elliptica* | Shade | 514 ± 59 | 2.96 ± 0.05 | 28.6 ± 3.0 | 25.5 ± 2.2 | 94.9 ± 6.8 | 0.4 ± 0.4 | 46.2 ± 1.0 | 18.1 ± 1.2 | **2.58 ± 0.22** | 49.5 |
| *Rhizophora mangle* | Shade | 561 ± 14 | 3.61 ± 0.02 | 34.0 ± 0.8 | 27.8 ± 0.9 | 133.6 ± 4.0 | n.d. | 2.6 ± 0.2 | 66.0 ± 1.9 | **0.04 ± 0.00** | 73.7 |
| Rubiaceae | | | | | | | | | | | |
| *Psychotria poeppigiana* | Sun (gap) | 431 ± 96 | 3.59 ± 0.17 | 35.6 ± 2.7 | 29.5 ± 1.4 | 118.2 ± 9.2 | n.d. | 41.9 ± 1.2 | 35.6 ± 5.2 | 1.23 ± 0.17 | 36.8 |
| | Shade | 338 ± 37 | 3.51 ± 0.12 | 37.2 ± 2.2 | 21.4 ± 2.1 | 121.8 ± 5.1 | n.d. | 49.2 ± 0.4 | 25.4 ± 1.8 | 1.96 ± 0.14 | 28.9 |
| Sapindaceae | | | | | | | | | | | |
| *Serjania mexicana* | Sun | 470 ± 30 | 4.01 ± 0.05 | 33.7 ± 1.1 | 45.7 ± 4.9 | 128.6 ± 7.4 | n.d. | 10.7 ± 2.7 | 71.9 ± 1.6 | 0.15 ± 0.04 | 83.7 |
| | Shade | 500 ± 42 | 3.46 ± 0.09 | 34.4 ± 1.2 | 24.0 ± 1.3 | 111.7 ± 3.6 | 2.5 ± 0.2 | 31.6 ± 1.8 | 40.4 ± 1.8 | 0.78 ± 0.05 | 75.3 |
| Simaroubaceae | | | | | | | | | | | |
| *Quassia amara* | Shade | 523 ± 21 | 3.08 ± 0.01 | 36.6 ± 0.2 | 14.2 ± 0.3 | 129.5 ± 1.3 | 3.9 ± 0.7 | 44.0 ± 0.3 | 26.1 ± 0.7 | 1.69 ± 0.04 | 31.1 |
| Theaceae | | | | | | | | | | | |
| *Freziera candicans* | Sun | 370 ± 31 | 4.32 ± 0.11 | 34.1 ± 1.1 | 58.1 ± 0.8 | 126.0 ± 2.5 | n.d. | 2.3 ± 0.1 | 80.7 ± 3.0 | 0.03 ± 0.00 | **183.2*** |
| | Shade | 438 ± 66 | 3.61 ± 0.09 | 29.7 ± 0.8 | 24.7 ± 0.7 | 108.9 ± 1.5 | n.d. | 6.1 ± 0.4 | 52.8 ± 4.4 | 0.12 ± 0.02 | **157.9** |
| Thymelaeaceae | | | | | | | | | | | |
| *Daphnopsis americana* | Shade | 605 ± 29 | 3.18 ± 0.09 | 40.7 ± 1.1 | 20.8 ± 0.7 | 144.1 ± 3.2 | 1.8 ± 0.3 | 0.9 ± 0.2 | 65.1 ± 1.4 | 0.01 ± 0.00 | 35.3 |
| Tiliaceae | | | | | | | | | | | |
| *Luehea seemannii* | Sun | 581 ± 53 | 3.03 ± 0.59 | 33.1 ± 5.4 | 44.8 ± 8.8 | 124.7 ± 7.1 | n.d. | 24.8 ± 4.9 | 50.8 ± 11.8 | 0.50 ± 0.07 | |
| | Shade | 543 ± 13 | 1.53 ± 0.04 | 26.8 ± 0.7 | 25.1 ± 1.4 | 106.3 ± 4.6 | n.d. | 22.8 ± 2.6 | 13.3 ± 1.4 | 1.72 ± 0.15 | 42.1 |
| Turneraceae | | | | | | | | | | | |
| *Turnera panamensis* | Shade | 404 ± 15 | 3.01 ± 0.05 | 34.7 ± 1.8 | 21.5 ± 1.5 | 127.5 ± 7.0 | 2.4 ± 1.2 | 0.4 ± 0.4 | 59.8 ± 2.7 | 0.01 ± 0.01 | 32.6 |
| Verbenaceae | | | | | | | | | | | |
| *Avicennia germinans* | Shade | 461 ± 37 | 3.35 ± 0.04 | 33.7 ± 1.2 | 32.2 ± 1.5 | 121.1 ± 3.3 | 0.6 ± 0.6 | 2.1 ± 0.2 | 63.9 ± 7.7 | 0.03 ± 0.00 | 67.4 |
| *Petrea aspera* | Shade | 516 ± 20 | 3.14 ± 0.05 | 34.3 ± 1.3 | 17.4 ± 0.6 | 122.0 ± 1.8 | 2.3 ± 0.5 | 47.9 ± 0.6 | 22.1 ± 0.9 | 2.18 ± 0.07 | 53.7 |

**Table 2.** (*continued*)

| Family and species | Leaf type | Chl *a*+*b* | Chl *a*/*b* | N | VAZ | L | Lx | α-Car | β-Car | α-/β-Car | LMA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Violaceae | | | | | | | | | | | |
| *Hybanthus prunifolius* | Shade | 348 ± 40 | 3.62 ± 0.05 | 23.6 ± 3.3 | 21.7 ± 7.3 | 105.1 ± 6.8 | n.d. | 52.2 ± 8.7 | 42.2 ± 10.6 | 0.75 ± 0.31 | 22.1 |
| Viscaceae | | | | | | | | | | | |
| *Dendrophthora ambigua* | Sun | 250 ± 2 | 4.48 ± 0.19 | 35.9 ± 0.9 | 85.2 ± 5.4 | 174.4 ± 9.5 | n.d. | n.d. | 56.7 ± 3.5 | | **243.2** |
| Vitaceae | | | | | | | | | | | |
| *Vitis tiliifolia* | Sun | 257 ± 3 | 3.88 ± 0.20 | 36.2 ± 1.3 | 63.2 ± 3.3 | 119.7 ± 0.1 | n.d. | 5.2 ± 0.5 | 75.1 ± 0.9 | 0.07 ± 0.01 | 74.7 |
| | Shade | 457 ± 23 | 3.08 ± 0.13 | 40.3 ± 2.2 | 26.2 ± 1.3 | 138.8 ± 3.0 | 5.3 ± 1.9 | 23.1 ± 1.8 | 46.8 ± 4.3 | 0.51 ± 0.09 | 66.8 |
| Vochysiaceae | | | | | | | | | | | |
| *Vochysia ferruginea* | Sun | 365 ± 27 | 4.55 ± 0.21 | 24.6 ± 1.6 | 18.9 ± 3.3 | 103.4 ± 3.9 | 3.0 ± 0.5 | 10.3 ± 1.9 | 67.0 ± 2.0 | 0.16 ± 0.03 | 83.7 |
| | Shade | 857 ± 20 | 3.53 ± 0.01 | 32.3 ± 1.0 | 25.4 ± 0.6 | 106.8 ± 0.8 | 6.5 ± 0.7 | 29.1 ± 1.1 | 51.2 ± 0.8 | 0.57 ± 0.01 | 116.8 |

*Inga* species (Fabaceae-Mimosoideae) studied previously (Matsubara *et al*. 2008). Very high amounts of Lx were detected in both sun and shade leaves of *Virola elongata* and *V. sebifera* (Myristicaceae), as has been reported for *V. surinamensis* (Matsubara *et al*. 2008). Intermediate levels of Lx (5–10 mmol mol Chl$^{-1}$) were found in shade leaves of 11 species and 36 species contained low levels of Lx (<5 mmol mol Chl$^{-1}$) in shade and/or sun leaves. In 31 species, Lx was not detectable.

In order to compare sun-acclimation responses of photosynthetic pigments in the two major life forms of tropical forest canopies, i.e. trees and lianas, data of sun leaves were combined for 12 tree species and nine liana species from seasonally dry forests (Table 3). In sun leaves lianas accumulated significantly larger amounts of the V-cycle pigments on a Chl basis than trees ($P \leq 0.001$). Levels of other pigments did not differ significantly between the two groups.

### Sun-shade leaf characteristics

Leaf dry mass, Chl *a*+*b* and carotenoid contents (all on a leaf area basis) were compared between shade and sun leaves (Fig. 1). Data are presented in box plots, illustrating species distribution for each leaf type. Generally, shade leaves had lower LMA than sun leaves (Fig. 1*A*; $P < 0.001$). Sun leaves of *Dendrophthora ambigua* (mistletoe, Viscaceae) and *Freziera candicans* (Theaceae) and shade leaves of *Myrcia sylvatica* (Myrtaceae) and *F. candicans* showed the highest LMA values for each leaf type. Shade leaves tended to contain higher amounts of Chl per unit leaf area than sun leaves (Fig. 1*B*; $P = 0.015$), but there was no significant difference in the level of total carotenoids (Fig. 1*C*).

The amounts of pigments on a leaf dry mass basis were plotted against the parameter of leaf structural trait, LMA, in Fig. 2. Although data for shade and sun leaves are strongly overlapping, those with very low LMA and high pigment contents on a mass basis mostly belong to shade leaves. Similar observations for Chl *a*+*b* and total carotenoids (Fig. 2*A*, *B*, respectively) indicate a common trend between LMA and accumulation of these photosynthetic pigments: the lower the LMA, the higher the pigment contents per leaf dry mass. In the lowest range of LMA, leaves of different species varied mainly in pigment contents and barely in LMA. *Vice versa*, in the lowest range of pigment contents, species variations were largely due to different LMA. Notably, all data, representing both leaf types of a broad range of tropical forest species, formed a single correlation between LMA and mass-based pigment levels.

Figure 3 depicts data for different carotenoids based on leaf area (Fig. 3*A*), Chl *a*+*b* (Fig. 3*B*) and leaf dry mass (Fig. 3*C*). On a leaf area basis, shade leaves, having more Chl (Fig. 1*B*), also contained more N than sun leaves (Fig. 3*A*). Likewise, α-Car accumulated more strongly in shade leaves, whereas VAZ and β-Car exhibited an opposite trend, i.e. higher in sun than in shade leaves. Regardless of the leaf type, species varied strongly in the amounts of L, or L plus Lx (LxL). When the data were expressed on a Chl basis (Fig. 3*B*), sun-shade patterns remained unchanged for VAZ, α- and β-Car whereas the differences between the two leaf types disappeared for N and variation was greatly reduced for LxL. The two highest values of LxL were found in shade leaves of *Vanilla planifolia* (Orchidaceae) and *Costus villosissimus* (Zingiberaceae) and sun leaves of

**Table 3. Pigment comparison between sun leaves of 12 tree species and nine liana species collected in seasonally dry forests**
Samples of sun leaves ($n = 3$, from individual leaves or leaflets) were collected in site 1 and 2 (Table 1). Pigment contents are given on a leaf area basis (μmol m$^{-2}$) for total chlorophylls (Chl *a*+*b*) and total carotenoids (Σ Caro) or on a Chl basis (mmol mol Chl *a*+*b*$^{-1}$) for different carotenoids. N, neoxanthin; VAZ, sum of the violaxanthin-cycle pigments; LxL, sum of lutein and lutein epoxide, α-Car, α-carotene; β-Car, β-carotene. Values are mean ± s.e. Means of VAZ are significantly different (*) between trees and lianas ($P \leq 0.001$ by Student *t*-test)

| | Leaf area basis (μmol m$^{-2}$) | | Chlorophyl basis (mmol mol Chl *a*+*b*$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| | Chl *a*+*b* | Σ Caro | N | VAZ* | LxL | α-Car | β-Car |
| Trees | 500 ± 56 | 140 ± 15 | 34 ± 2 | 41 ± 5 | 128 ± 10 | 12 ± 5 | 68 ± 7 |
| Lianas | 457 ± 31 | 135 ± 8 | 34 ± 1 | 61 ± 3 | 133 ± 9 | 10 ± 1 | 67 ± 2 |

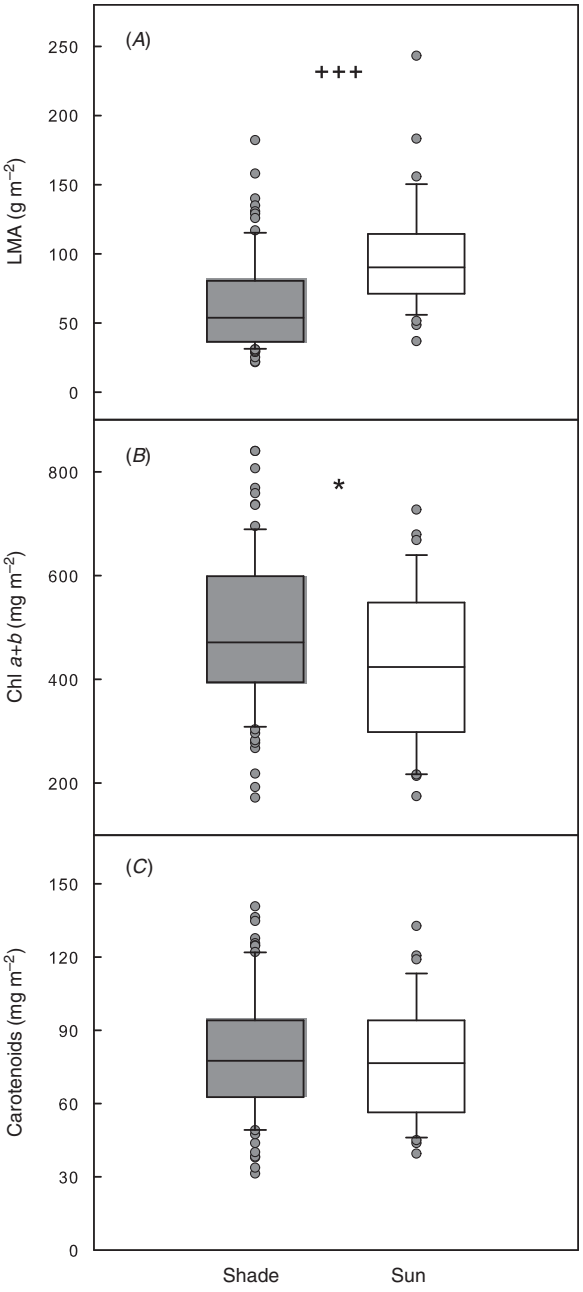



**Fig. 1.** (*A*) Leaf dry mass per area (LMA), (*B*) total chlorophyll (Chl *a*+*b*), and (*C*) total carotenoid contents per area of shade (grey boxes) and sun leaves (white boxes). Mean values from individual species were used in the analysis. The lower boundary of each box indicates the 25th percentile, a line within the box marks the median, and the upper boundary of the box indicates the 75th percentile. Whiskers below and above the box denote the 10th and 90th percentiles, respectively. Symbols beyond whiskers represent outlying data. Shade and sun datasets were significantly different for LMA ($P < 0.001$ by rank sum test, significance denoted by three plus signs) and for Chl *a*+*b* ($P = 0.015$ by *t*-test, significance denoted by an asterisk). For methods of statistical analysis, see 'Materials and methods'.

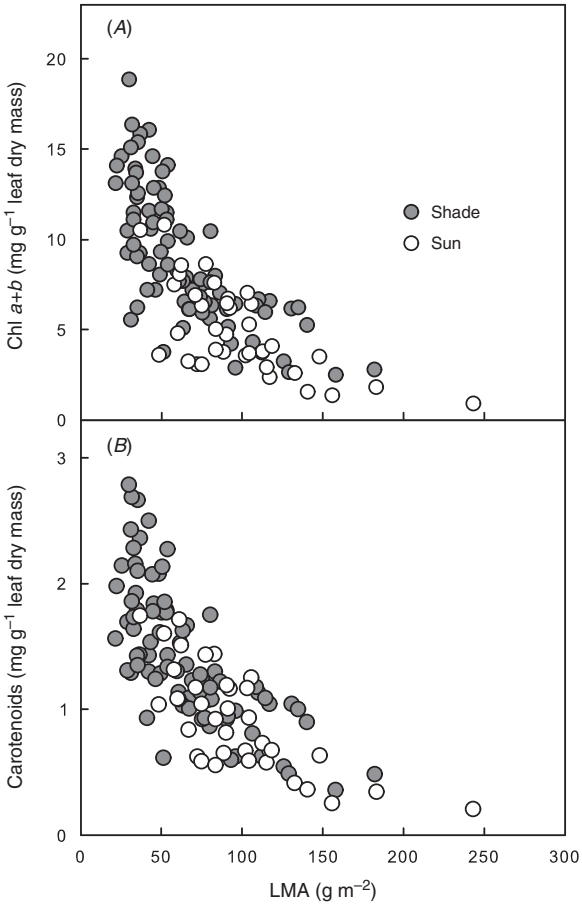



**Fig. 2.** Relationship between leaf dry mass per area (LMA) and (*A*) total chlorophyll (Chl *a*+*b*) and (*B*) total carotenoid contents on a leaf dry mass basis in shade and sun leaves. Each symbol represents a mean value ($n = 3$ for pigments, $n = 2$ for LMA) of each species and leaf type. Open symbols, sun leaves; closed symbols, shade leaves.

*Vanilla planifolia* and *Clusia pratensis* (Clusiaceae) (see also Table 2).

The amounts of different carotenoids were also compared on a leaf dry mass basis (Fig. 3*C*). Shade leaves generally had greater amounts of N as well as LxL per unit leaf dry mass than sun leaves. The species with extremely high values of LxL on a leaf dry mass basis were *Daphnopsis americana* (Thymelaeaceae), *Xiphidium caeruleum* (Haemodoraceae) and *Quassia amara* (Simaroubaceae) for shade leaves and *Psychotria poeppigiana* (Rubiaceae) for sun leaves. On a dry mass basis, the sun-shade difference in α-Car became even more evident than was seen on a leaf area or Chl *a*+*b* basis, with the levels in sun leaves extremely low in most species. Further, shade and sun leaves were found to be comparable in terms of VAZ and β-Car relative to leaf dry mass, contrary to the typical sun-shade behaviour of these pigments on a leaf area or Chl *a*+*b* basis (Fig. 3*A*–*C*). Shade leaves of *D. americana* and

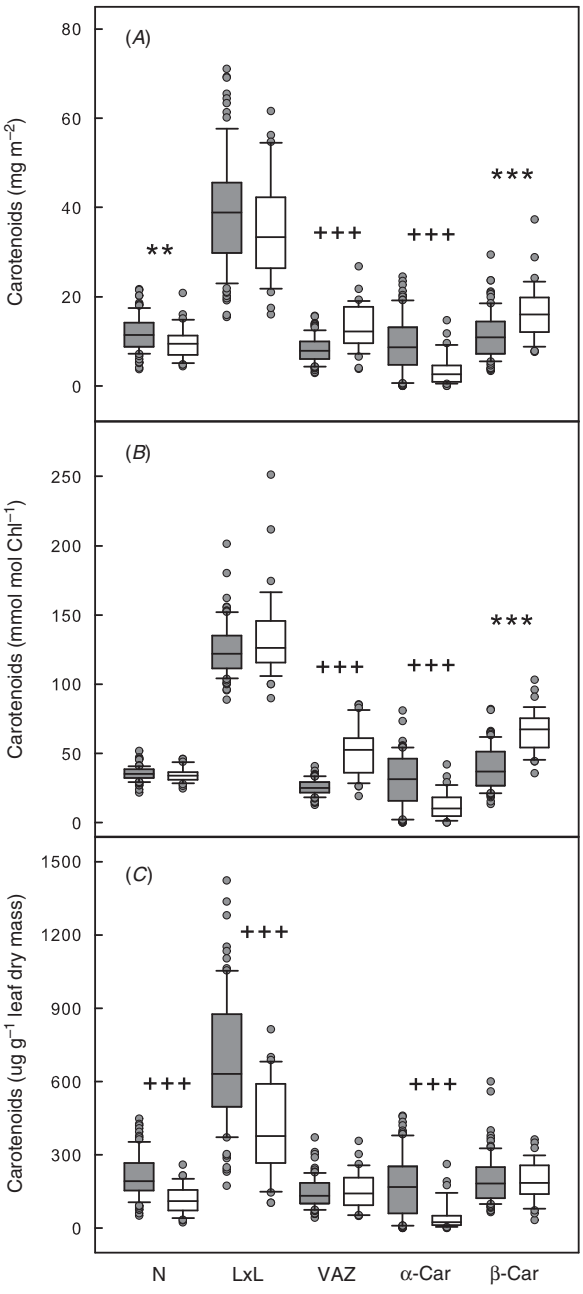



**Fig. 3.** Contents of different carotenoids in shade (grey boxes) and sun leaves (white boxes) based on (*A*) leaf area, (*B*) Chl *a*+*b*, and (*C*) leaf dry mass. N, neoxanthin; LxL, sum of lutein and lutein epoxide; VAZ, sum of viola-, anthera- and zeaxanthin; α-Car, α-carotene; β-Car, β-carotene. For explanation of box plots, see legend to Fig. 1. Asterisks and plus signs indicate significant differences between shade and sun leaves tested by *t*-test and rank sum test, respectively. *** and +++, $P < 0.001$; **, $P = 0.006$.

*Scleria latifolia* (Cyperaceae) contained extremely high levels of β-Car on a dry mass basis.

### *Interrelation between carotenoids in shade and sun leaves*

The relationship between carotenoids synthesised in the β,β- and β,ε-branch is depicted in Fig. 4. On a leaf area basis (Fig. 4*A*), both shade and sun leaves exhibited large variations in the levels of β,β- and β,ε-carotenoids. Nevertheless, for a given amount of β,ε-carotenoids, sun leaves tended to accumulate more β,β-carotenoids than shade leaves. Very high levels of β,ε-carotenoids were found mostly in shade leaves. The apparently linear correlation from lower left to upper right in Fig. 4*A* probably reflects differences in LMA among species (Fig. 1*A*). When expressed on a Chl basis (Fig. 4*B*), the sum of β,ε-carotenoids was rather uniform among sun and shade leaves, centred at around 150 mmol mol Chl$^{-1}$, except for some species showing extreme values (>200 mmol mol Chl$^{-1}$). Higher β,β-carotenoid levels in sun compared with shade leaves are clearly seen on a leaf area basis as well as on a Chl basis (Fig. 4*A*, *B*).

Figure 5 shows the relationship between the ratios β,ε-/β,β-carotenoids and α-/β-Car. Data from sun leaves were confined within the region of low to intermediate values of β,ε-/β,β-carotenoids (~0.5 to 1.5) and low α-/β-Car (mostly <0.5). Among shade leaves, in contrast, pronounced variability was found for both β,ε-/β,β-carotenoids and α-/β-Car. There was a linear correlation between the two parameters, which is stronger for shade than for sun leaves. The slope of the regression line of shade leaves (1.75) suggests that variations in β,ε-/β,β-carotenoids are accompanied by larger differences in α-/β-Car. In comparison, sun leaves exhibited smaller variations in α-/β-Car than in β,ε-/β,β-carotenoids (slope 0.79).

The striking divergence in α-/β-Car found in sun and shade leaves of different species was largely attributable to substitution between these two carotenes (Fig. 6). In leaves with high amounts of β-Car, the α-Car levels were low and *vice versa*. The sum of α- and β-Car varied little between sun and shade leaves (see legend to Fig. 6). Although a wide range of α-Car levels was found in shade leaves (from zero to ~80 mmol mol$^{-1}$ Chl), sun leaves rarely reached α-Car levels >45 mmol mol Chl$^{-1}$ (see also Table 2).

Figure 7 illustrates the relationship between α-/β-Car and VAZ. The sum of the V-cycle pigments reached highest levels in sun leaves having low α-/β-Car (< 0.5). There was little variation in VAZ among shade leaves, regardless of α-/β-Car. It is noteworthy that all data points, without a single exception, were scattered either at the bottom of the panel along the *x*-axis (mostly shade leaves) or on the left-hand side along the *y*-axis (mostly sun leaves), indicating that high α-/β-Car and a large VAZ pool did not co-exist in any of the leaves examined.

### *Lx and V cycles in* Virola elongata *in situ*

Given the high Lx contents in sun leaves of *Virola* species (Table 2; Matsubara *et al.* 2008), we examined for canopy leaves of *V. elongata* whether the Lx and V cycles operate in parallel in a diel manner *in situ* (Fig. 8). In the early morning, the Lx pool size was similar in sun and shade leaves but that of V differed markedly. As PAR rose to ~2000 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 8*A*), a large proportion of

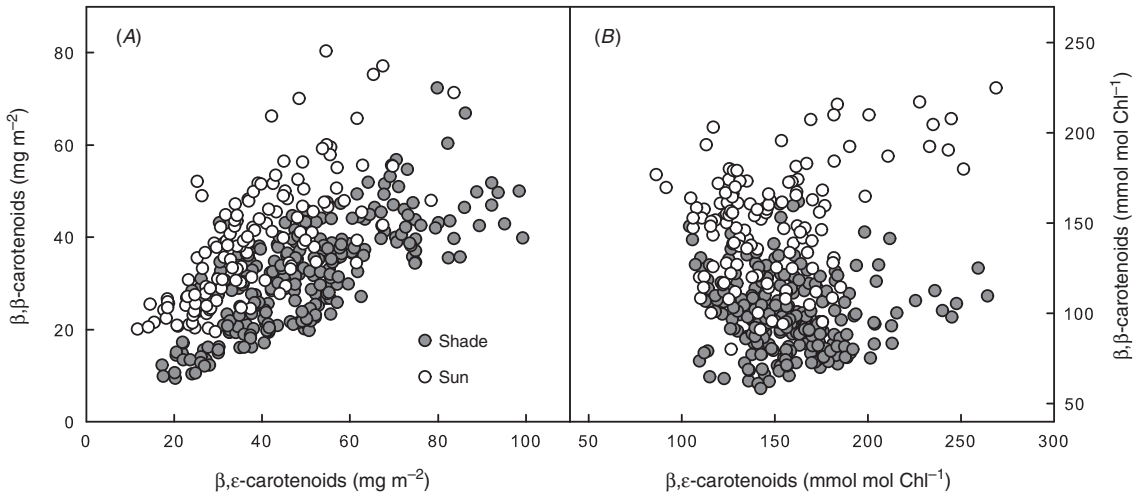



**Fig. 4.** Relationship between the sum of β,β- and β,ε-carotenoids in shade and sun leaves. Data are shown on a (*A*) leaf area, and (*B*) Chl *a*+*b* basis. Symbols represent individual leaves or leaflets. Open symbols, sun leaves; closed symbols, shade leaves.

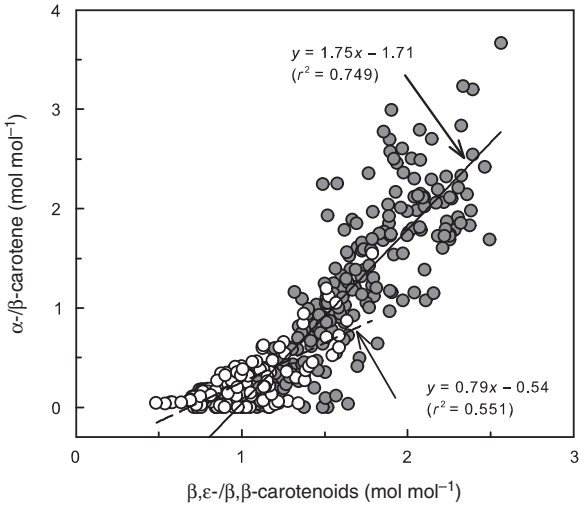



**Fig. 5.** Relationship between the ratios β,ε-/β,β-carotenoids and α-/β-carotene. Symbols represent individual leaves or leaflets. Open symbols, sun leaves; closed symbols, shade leaves. Solid line, linear regression of shade leaves; dashed line, linear regression of sun leaves.

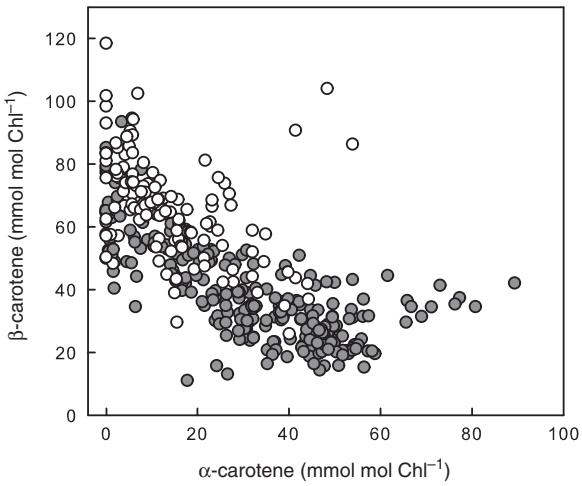



**Fig. 6.** Relationship between the contents of α- and β-carotene. Data are given on a Chl *a*+*b* basis. Symbols represent individual leaves or leaflets. Open symbols, sun leaves; closed symbols, shade leaves. The sum of α- and β-carotene was 81 ± 15 and 70 ± 13 mmol mol$^{-1}$ Chl for sun and shade leaves, respectively.

Lx and V was de-epoxidised rapidly in sun leaves (Fig. 8*B*), accompanied by a significant reduction in $F_v/F_m$ (see legend to Fig. 8). No substantial xanthophyll turnover was detected in shade leaves, for which PAR remained <100 µmol photons m$^{-2}$ s$^{-1}$ and $F_v/F_m$ stayed constant (Fig. 8*A*, *C*, see legend to Fig. 8 for $F_v/F_m$). Apparent fluctuations of Lx and V in shade leaves presumably resulted from variations between individual leaves collected at different time points.

In sun leaves detached and dark-adapted at around midday, ~50% of V was restored by 1440 hours and 1710 hours (grey symbols in Fig. 8*B*), corresponding to a partial recovery of $F_v/F_m$ in these leaves (see legend to Fig. 8). Restoration of Lx occurred more slowly than that of V in the detached and dark-adapted sun leaves, but both Lx and V were found to be fully restored by the next morning in sun leaves *in situ*. Obviously, both xanthophyll cycles were operating in a 24-h cycle in sun leaves of *V. elongata*, albeit with different restoration (epoxidation) kinetics. In this canopy experiment performed during the dry season, the amounts of Lx found in sun leaves early in the morning were considerably lower than those in dark-adapted sun leaves collected from the same tree during the rainy season (Table 2).

## Discussion

### *Carotenoid contents as a leaf functional trait in neotropical forest species*

Large-scale studies of plants from different biomes indicate a universal correlation between different leaf functional traits, such as LMA, leaf life-span, rates of photosynthesis, respiration, and nitrogen content (Reich *et al*. 1997; Wright *et al*. 2004). Correlations are generally stronger when traits are expressed on a leaf dry mass basis, which emphasises leaf economics (cost and benefit) than on a leaf area basis which considers fluxes of PAR, $CO_2$, and $H_2O$. For example, rates of photosynthesis per unit dry mass decline as LMA increases. We observed a similar trend between leaf chlorophyll and carotenoid contents, respectively, and LMA (Fig. 2). At low values of LMA, characteristic of shade leaves (Fig. 1*A*), pigment content was particularly sensitive to changes in LMA, and pigment content decreased to a lesser extent as LMA increased to higher values (Fig. 2). Data points for sun and shade leaves from diverse tropical plant taxa fitted the same relationship (Fig. 2), an observation which is not necessarily true for relationships between photosynthesis rate and LMA, or leaf nitrogen content and LMA in sun and shade leaves (Santiago and Wright 2007).

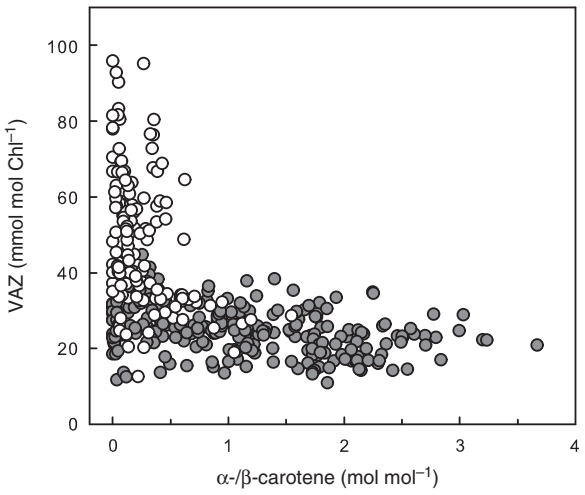



**Fig. 7.** Relationship between the ratio α-/β-carotene and the pool size of the violaxanthin-cycle pigments (VAZ) given on a Chl *a*+*b* basis. Symbols represent individual leaves or leaflets. Open symbols, sun leaves; closed symbols, shade leaves.

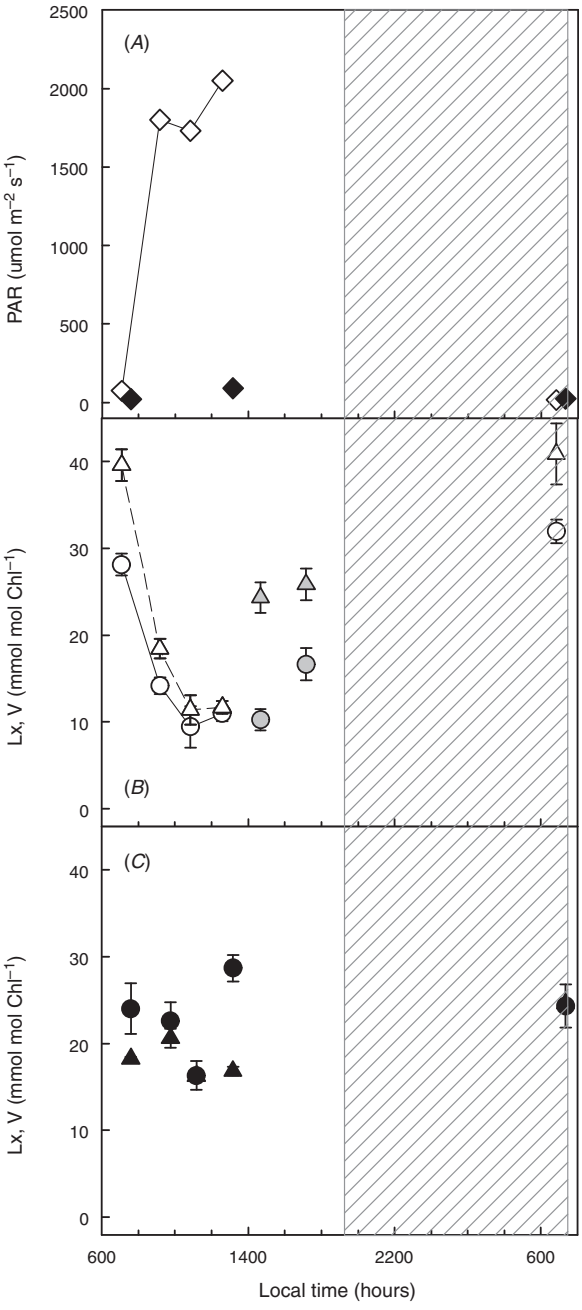
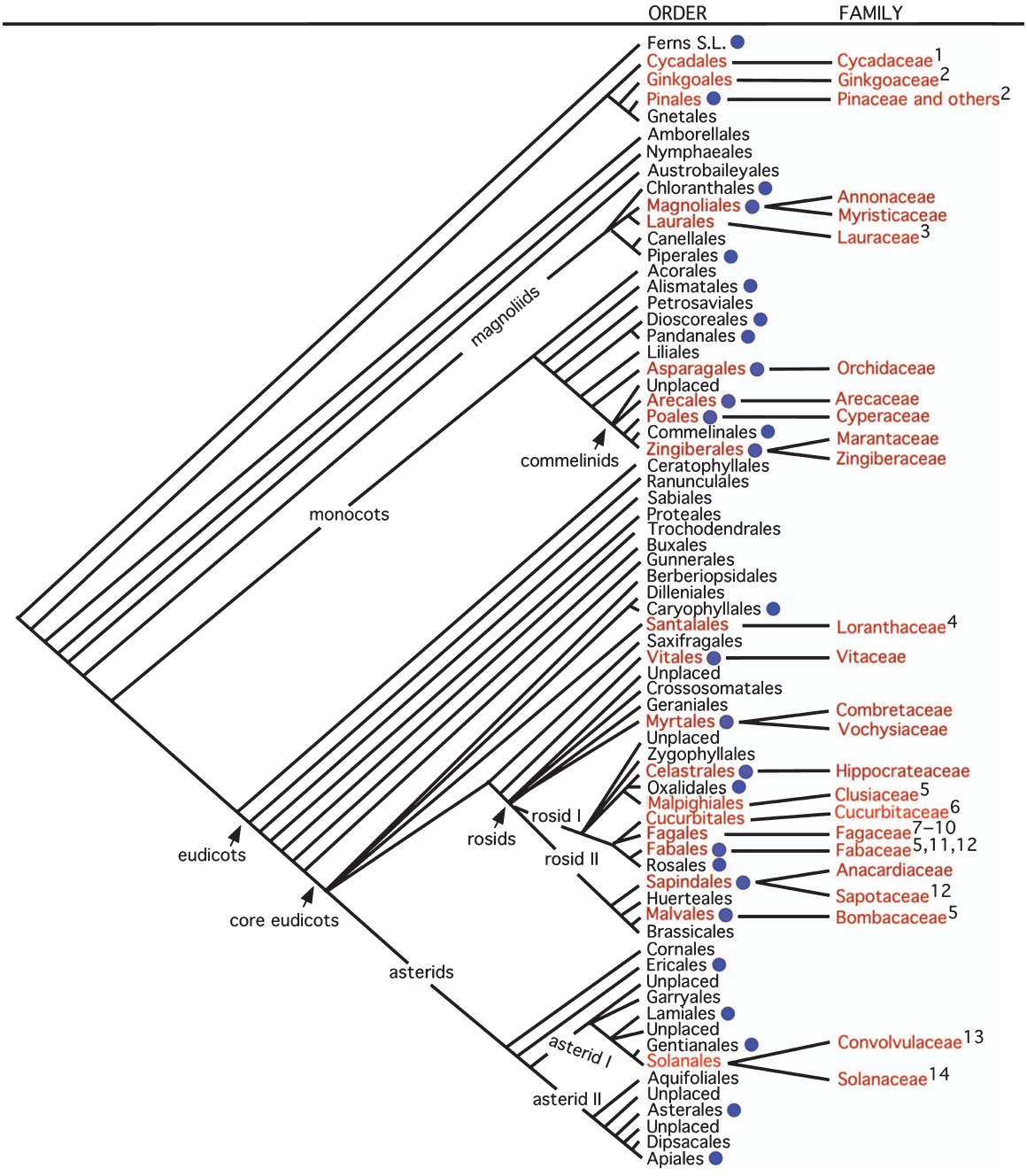



**Fig. 8.** Diel changes in contents of lutein epoxide (Lx, circles) and violaxanthin (V, triangles) in sun (open symbols, *B*) and shade leaves (closed symbols, *C*) of *Virola elongata*. The PAR incident on leaves (diamonds) is shown in (*A*). Error bars (shown when larger than symbols) indicate s.e. ($n = 5$). Different sets of leaves were used at the four sampling times from morning to midday. The time courses from morning to midday are indicated for sun leaves by solid lines (PAR and Lx) or a dashed line (V). The hatched area indicates the nighttime. After measurements at 1240 hours, five sun leaves were detached and dark-adapted at ambient temperature. The grey symbols in (*B*) show the contents of pigments found in these detached sun leaves after 2 and 4.5 h of dark adaptation. The $F_v/F_m$ (means ± s.e.; $n = 5$) measured in sun leaves was 0.767 ± 0.009 at 0705 hours (first morning), 0.700 ± 0.008 at 1240 hours, 0.735 ± 0.005 after 4.5 h dark adaptation of detached leaves and 0.785 ± 0.005 at 0650 hours (second morning *in situ*). The $F_v/F_m$ values in shade leaves were 0.808 ± 0.001 at 0750 hours (first morning), 0.803 ± 0.003 at 1310 hours and 0.814 ± 0.001 at 0720 hours (second morning).




[1]Cardini *et al.* 2006; [2]Czeczuga 1986; [3]Esteban *et al.* 2007; [4]Matsubara *et al.* 2003; [5]Matsubara *et al.* 2008; [6]Schwickert JA, Matsubara S, unpublished; [7]García-Plazaola *et al.* 2002; [8]García-Plazaola *et al.* 2004; [9]Llorens *et al.* 2002; [10]Munné-Bosch *et al.* 2004; [11]Watson *et al.* 2004; [12]Krause GH, Gallé A, Gademann R, Winter K, unpublished; [13]Bungard *et al.* 1999; [14]Rabinowitch *et al.* 1975.

**Fig. 9.** Occurrence of moderate to high Lx levels within the phylogenetic tree of vascular plants. The scheme has been adopted after the Missouri Botanical Garden phylogeny website (www.mobot.org). Blue dots mark plant orders, in which at least one species has been tested in the present study; when at least one species contained >5 mmol Lx mol $Chl^{-1}$ in shade leaves (or both shade and sun leaves), orders and families have been marked in red. Previous reports on significant amounts of Lx in leaves or other green tissues have also been considered by red marking (references given in the footnote).

Although high Chl *a*+*b* content and low LMA (Fig. 1*A*, *B*) are consistent with a primary demand of shade leaves for light harvesting, the economics of pigment accumulation has rarely been studied for carotenoids, presumably due to their small contribution to leaf dry mass (<0.3%, Fig. 2*B*). The results in Fig. 2 raise the question whether, as is the case with Chl, investment in carotenoids is more important for shade than for sun leaves. On a leaf area basis, total carotenoid content was very similar in sun and shade leaves (Fig. 1*C*). Based on leaf dry mass, sun-shade differences in N and LxL were highly significant, and those of VAZ and β-Car were found to be significant only on a leaf area or Chl basis (Fig. 3). Both N and L are integral components of LHCII, the major light-harvesting antenna of PSII (Kühlbrandt *et al*. 1994; Liu *et al*. 2004). Although N and L may fulfil photoprotective functions in LHCII (Niyogi *et al*. 1997; Pogson *et al*. 1998; Dall'Osto *et al*. 2007*a*, 2007*b*; Ruban *et al*. 2007; Matsubara *et al*. 2008), selective mass-based accumulation of these pigments in shade leaves seems to be primarily coupled with accumulation of LHCII for light harvesting (Björkman 1981; Anderson *et al*. 1988), as was seen from enhanced accumulation of mass-based Chl and total carotenoids (Fig. 2) as well as generally lower values of Chl *a*/*b* in shade leaves compared with sun leaves of the same species (Table 2). For the sun-shade differences in VAZ and β-Car, in contrast, incident PAR flux and/or high-light stress caused by adverse environmental conditions are probably the major determinants because the variations were seen only on a leaf area or Chl basis (Fig. 3). The finding of a higher VAZ pool size (+50% based on Chl) in sun leaves of lianas compared with sun leaves of trees (Table 3) suggests that lianas possess a greater photoprotection capacity, possibly because they experience stronger high-light stress. This is in agreement with the observations by Santiago and Wright (2007) that outer-canopy sun leaves of lianas exhibit lower photosynthetic capacities than sun leaves of trees having similar LMA.

The striking similarity of shade and sun leaves with respect to mass-based VAZ and β-Car (Fig. 3*C*) is puzzling as it suggests that investment of leaves in these photoprotective pigments is little affected by growth light environment. This contrasts the investment into light-harvesting pigments (Chl, N, LxL and α-Car) which is strongly influenced by the light environment (Figs 2, 3*C*). Overall, however, the general trend of the relationship between mass-based photosynthetic pigments (Chl and carotenoids) and LMA across both sun and shade leaves (Fig. 2) suggests that trade-offs among these traits are under similar leaf functional constraints regardless of species and growth environment. The distinct sun-shade pattern of α-Car was independent of whether it is expressed on an area, dry mass, or Chl basis, a result which reflects the general preference of β-Car over α-Car under high irradiance.

### *Sun-shade acclimation and adaptation in carotenoid biosynthetic pathways*

The balance shift between the β,ε- and β,β-branch of the carotenoid biosynthetic pathway (Fig. 4) seems to be a universal response in leaves of neotropical forest species to light environments. Based both on leaf area (Fig. 4*A*) and Chl (Fig. 4*B*), the data show a clear trend to higher β,β-carotenoids in sun compared with shade leaves, whether it is adaptational or acclimatory. Although values of β,ε-/β,β-carotenoids varied hugely among shade leaves of different species, it was rather uniform, mostly ≤1, among sun leaves (Fig. 5). The same pattern was found for α-/β-Car, i.e. the balance between the first products of the two branches, which correlates positively and linearly with the balance between the whole branches, i.e. β,ε-/β,β-carotenoids (Fig. 5). A similar correlation between β,ε-/β,β-carotenoids and α-/β-Car has been observed in a previous study on sun and shade leaves of different *Inga* species (Matsubara *et al*. 2008). The confined data distribution of sun leaves in Fig. 5 suggests selection pressure by high light, constraining an increase in α-/β-Car and β,ε-/β,β-carotenoids. The fact that there was no single leaf having a large Chl-based pool of VAZ and high α-/β-Car at the same time (Fig. 7) supports this notion. The absence of such stress under shade conditions may then allow the large interspecific variation of α-/β-Car and β,ε-/β,β-carotenoids (Fig. 5).

Furthermore, our data suggest that α- and β-Car substitute each other in most cases (Fig. 6), minimising variations in the sum of the two carotenes (see legend to Fig. 6). The substitution supposedly takes place in the core complexes of PSII and in the core and antennae of PSI (Matsubara *et al*. 2007). Accumulation of α-Car has been found in shade leaves of many different plants (e.g. Thayer and Björkman 1990; Demmig-Adams and Adams 1992; Siefermann-Harms 1994; Demmig-Adams 1998), including neotropical forest species (Königer *et al*. 1995; Krause *et al*. 2001, 2003, 2004; Matsubara *et al*. 2008; Table 2). Based on enhanced accumulation in shade environments, a possible function of α-Car in improving light harvesting has been proposed (Krause *et al*. 2001). Besides contributing to light harvesting, β-Car is thought to exert photoprotection in the core antennae by quenching of triplet-state Chl *a* and singlet-state dioxygen (Frank and Cogdell 1996; Scheer 2003) and in the PSII reaction centre by singlet-state dioxygen scavenging and mediating cyclic electron transfer (Telfer 2002). Differential functions of α- and β-Car in light harvesting and photoprotection need to be verified in the future.

Several species, however, did exhibit low α-Car levels in shade leaves whereas sun leaves seemed to have a strict preference for β-Car (Figs 5–7; Table 2). This suggests that a high α-/β-Car is not a general requirement for shade acclimation or adaptation. In those species, where α-Car is high in the shade, α-Car resembles Lx, which also accumulates to high levels under shaded environments, although the occurrence of Lx in tropical forest species is less frequent than of α-Car (Table 2). Since α-Car is a precursor of L, the most abundant and universal xanthophyll in leaves, all higher plants must have the enzymes necessary for α-Car synthesis. In *Arabidopsis thaliana*, which does not accumulate α-Car naturally, absence of the *lut5* gene product, a heme β-ring hydroxylase, leads to accumulation of α-Car (Kim and DellaPenna 2006; Fiore *et al*. 2006). It is possible that β-ring hydroxylation in the β,ε-branch is involved in the regulation of α-/β-Car in shade and sun leaves of many higher plant species in response to growth irradiance. In addition, regulation of ε-ring cyclisation at the branching point of carotenoid biosynthesis (Pogson *et al*. 1996; Pogson and Rissler 2000) and β-ring hydroxylation in the β,β-branch (Davison *et al*. 2002) may

adjust β,ε-/β,β-carotenoids and VAZ pool, respectively, to light environments.

### *Occurrence of Lx among vascular plant taxa*

Among the 86 neotropical forest species collected in the present study, Lx was found in a broad range of rather unrelated plant taxa, as depicted in the scheme of the phylogenetic tree (Fig. 9), which also considers previous reports on occurrence of significant amounts of Lx. As Lx levels in leaves are subjected to both developmental and environmental control (García-Plazaola *et al*. 2007), the Lx values shown in Table 2 do not necessarily reflect the species capacity to accumulate this pigment in leaves. However, given the extended knowledge from the present and several previous studies (e.g. the references in Fig. 9), it is obvious that the ability to accumulate relatively high amounts of Lx has evolved independently among different plant taxa (García-Plazaola *et al*. 2004).

Our survey, focussed on neotropical forest species, indicates that occurrence of Lx is a rather common phenomenon in shade environments; 19 species (22%) had >5 mmol Lx mol $Chl^{-1}$, and eight species (9%) had >10 mmol Lx mol $Chl^{-1}$ in shade and/or sun leaves (Table 2). Analysing different life forms separately, four herbs, three lianas and 12 trees/shrubs contained >5 mmol Lx mol $Chl^{-1}$ in their leaves (22, 20 and 23% of the species for each life form, respectively). None of the herbs accumulated Lx to >10 mmol mol $Chl^{-1}$ whereas a liana and seven trees/shrubs did. Thus, Lx accumulation (>5 mmol mol $Chl^{-1}$) can be found rather widely and irregularly among different plant taxa and life forms, with a probability of ~20% for neotropical forest species, whereas higher Lx levels (>10 mmol mol $Chl^{-1}$) occur less frequently but still with a probability of ~10% for trees and shrubs in Panamanian forests. It is not known whether these numbers are higher or lower with respect to other habitats and vegetation types. Nevertheless, the wide distribution of Lx in tropical forest species implies functional significance in these plants.

Although the occurrence of Lx remains enigmatic both taxonomically and ecologically, extremely high Lx, so far, appears to be confined to tree species in a small number of orders. To these belong the two closely related magnoliid orders, Magnoliales and Laurales (Fig. 9), with high Lx contents in species of Myristicaceae and Lauraceae families (Table 2; Esteban *et al*. 2007, 2008; Matsubara *et al*. 2008), as well as Fabales in the clade of rosid I, with strong Lx accumulation in species of the Fabaceae-Mimosoideae family (Table 2; Matsubara *et al*. 2008). In the case of *Inga* species (Fabaceae-Mimosoideae), Lx is quickly de-epoxidised upon sunlight exposure but is restored very slowly in the shade (Matsubara *et al*. 2005, 2008), resulting in distinct sun-shade patterns of Lx levels, i.e. being much higher in shade than in sun leaves. Operation of this slowly reversible Lx cycle has been associated with shade-to-sun and sun-to-shade acclimation in long-lived leaves of *Inga* trees, wherein high Lx ensures efficient light harvesting in shade leaves and high L confers increased photoprotection in sun leaves (Matsubara *et al*. 2005, 2007, 2008), in addition to the well established function of the V cycle. Very high levels of Lx found in shade as well as sun leaves of *Virola* species (Table 2; Matsubara *et al*. 2008) represent a unique situation, in which daily sun exposure does not seem to limit Lx accumulation in sun leaves. The data indicate that occurrence of high Lx levels in different taxa (Fig. 9) do not necessarily signify the same regulation of the Lx-cycle operation.

### *Operation of the Lx cycle in* Virola elongata

In species, in which kinetics of post-illumination Lx restoration have so far been investigated, Lx was restored generally more slowly than V. This applies, for example, to chloroplast-containing stems of *Cuscuta reflexa* (Convolvulaceae) (Bungard *et al*. 1999; Snyder *et al*. 2005) and leaves of mistletoes (Loranthaceae) (Matsubara *et al*. 2001), *Quercus robur* (Fagaceae) (García-Plazaola *et al*. 2002), *Persea americana* (Lauraceae) (Esteban *et al*. 2008) and *Inga* species (Fabaceae) (Matsubara *et al*. 2005, 2008). These observations led to the conclusion that sun-shade Lx patterns commonly found among Lx-cycle plants could result from slow L→Lx epoxidation, with the extent of slowness differing between species but always limiting the Lx restoration when daily Lx→L de-epoxidation exceeds the capacity of daily L→Lx epoxidation.

The diel canopy experiment conducted on a *V. elongata* tree showed 60 and 70% de-epoxidation for Lx and V, respectively, in sun leaves in the strong daylight followed by full overnight restoration for both pigments (Fig. 8*B*). Hence, a large part of the Lx pool found in sun leaves of *V. elongata* is engaged in the xanthophyll cycling on a daily basis in parallel with V. Dark treatment of detached sun leaves in the afternoon revealed slower onset of restoration for Lx compared with V, with a delay of several hours (Fig. 8*B*). Similar situations have been reported for *Cuscuta reflexa* (Bungard *et al*. 1999) and mistletoe *Amyema miquelii* (Matsubara *et al*. 2001). The equally high Lx contents in sun and shade leaves of *Virola* plants, as opposed to marked sun-shade differences in Lx levels in other plants including mistletoes, may, therefore, suggest a large capacity of *Virola* sun leaves to restore Lx even though Lx restoration starts later than V restoration. At present, little is known about the regulation of epoxidation in the operation of the V cycle, and even less in the Lx cycle. Altered substrate specificity of the epoxidase enzyme to L has been suggested as a factor responsible for accumulation of Lx (Matsubara *et al*. 2003). Different binding affinities of light-harvesting complexes to L and Lx (Matsubara *et al*. 2007) could also play a role in distinct operation of the Lx cycle in different species. Clearly, more investigations are needed to elucidate the mechanism and physiological regulation of epoxidation in the Lx cycle. Species having extremely high levels of Lx with contrasting kinetics of Lx restoration, such as *Virola* and *Inga*, offer promising systems to address these questions. In particular, the possible function of the Lx cycle in leaves of *Virola* species, in which the levels of Lx and L are uncoupled from the marked sun-shade acclimation of VAZ and α-/β-Car (Table 2; Fig. 7), needs to be explored in future studies.

## Conclusion

Overall, our survey provided a new insight into the sun/shade balance between the various pigments of the two carotenoid biosynthesis branches and showed a wide distribution of α-

Car and Lx among taxa of tropical forest plants. The study of *Virola elongata* demonstrated for the first time a fully reversible diel operation of the Lx cycle in parallel with the V cycle in sun leaves containing comparable levels of Lx and V.

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## 6.2 Continuous Turnover of Carotenes and Chlorophyll *a* in Mature Leaves of Arabidopsis Revealed by $^{14}CO_2$ Pulse-Chase Labeling (Beisel et al., 2010)

# Continuous Turnover of Carotenes and Chlorophyll *a* in Mature Leaves of Arabidopsis Revealed by $^{14}CO_2$ Pulse-Chase Labeling[OA]

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Carotenoid turnover was investigated in mature leaves of Arabidopsis (*Arabidopsis thaliana*) by $^{14}CO_2$ pulse-chase labeling under control-light (CL; 130 $\mu$mol photons $m^{-2}$ $s^{-1}$) and high-light (HL; 1,000 $\mu$mol photons $m^{-2}$ $s^{-1}$) conditions. Following a 30-min $^{14}CO_2$ administration, photosynthetically fixed $^{14}C$ was quickly incorporated in $\beta$-carotene ($\beta$-C) and chlorophyll *a* (Chl *a*) in all samples during a chase of up to 10 h. In contrast, $^{14}C$ was not detected in Chl *b* and xanthophylls, even when steady-state amounts of the xanthophyll-cycle pigments and lutein increased markedly, presumably by de novo synthesis, in CL-grown plants under HL. Different light conditions during the chase did not affect the $^{14}C$ fractions incorporated in $\beta$-C and Chl *a*, whereas long-term HL acclimation significantly enhanced $^{14}C$ labeling of Chl *a* but not $\beta$-C. Consequently, the maximal $^{14}C$ signal ratio between $\beta$-C and Chl *a* was much lower in HL-grown plants (1:10) than in CL-grown plants (1:4). In *lut5* mutants, containing $\alpha$-carotene ($\alpha$-C) together with reduced amounts of $\beta$-C, remarkably high $^{14}C$ labeling was found for $\alpha$-C while the labeling efficiency of Chl *a* was similar to that of wild-type plants. The maximum $^{14}C$ ratios between carotenes and Chl *a* were 1:2 for $\alpha$-C:Chl *a* and 1:5 for $\beta$-C:Chl *a* in CL-grown *lut5* plants, suggesting high turnover of $\alpha$-C. The data demonstrate continuous synthesis and degradation of carotenes and Chl *a* in photosynthesizing leaves and indicate distinct acclimatory responses of their turnover to changing irradiance. In addition, the results are discussed in the context of photosystem II repair cycle and D1 protein turnover.

Carotenoids are classified as accessory pigments in photosynthesis because they augment light harvesting in the blue spectral region by transferring the absorbed light energy to chlorophyll (Chl). However, the universal occurrence of carotenoids in photosynthetic cells, from bacteria to higher plants, indicates their essential roles, rather than mere accessory roles, in photosynthesis. Under excess light, carotenoids provide protection against photooxidative damage by facilitating dissipation of excitation energy from singlet- or triplet-state Chl and scavenging highly reactive singlet oxygen, which is generated through interaction between triplet excited Chl and oxygen (Demmig-Adams, 1990; Müller et al., 2001). These photoprotective functions make carotenoids indispensable for oxygenic photosynthesis, as demonstrated by lethal effects of inhibitors of carotenoid biosynthesis in plants (Bramley, 1993). Regulation of light harvesting and photoprotection by carotenoids requires their close proximity as well as the proper orientation to Chl molecules in pigment-protein complexes of PSI and PSII. Furthermore, a small fraction of non-protein-bound carotenoids serves as antioxidants in the lipid phase of photosynthetic membranes (Havaux and Niyogi, 1999; Havaux et al., 2004) and influences the structure and fluidity of the lipid bilayer (Gruszecki and Strzałka, 2005). Despite these and other lines of defense, the PSII reaction center polypeptide D1, and to a lesser extent also D2, undergo frequent photooxidative damage and repair in the light (Melis, 1999; Baena-González and Aro, 2002). When the repair process cannot keep up with the rate of photodamage, the overall quantum yield of PSII declines.

Carotenoids are derived from isoprenoid precursors in plastids (for reviews on carotenoid biosynthesis in plants, see Lichtenthaler, 1999; Hirschberg, 2001; DellaPenna and Pogson, 2006; Giuliano et al., 2008; Tanaka et al., 2008; Cazzonelli and Pogson, 2010). Following the formation of linear $C_{40}$ lycopene, the pathway splits into two branches of major cyclic carotenoids: the $\beta,\beta$-branch gives rise to $\beta$-carotene ($\beta$-C) having two $\beta$-rings, whereas the $\beta,\varepsilon$-branch leads to formation of $\alpha$-carotene ($\alpha$-C) having one $\beta$-ring and one $\varepsilon$-ring. Hydroxylation of $\beta$-C and $\alpha$-C produces the xanthophylls zeaxanthin (Z) and lutein (L), respectively. In the $\beta,\beta$-branch, epoxidation of the $\beta$-rings of Z results in successive synthesis of antheraxanthin (A) and violaxanthin (V); subsequently, V can

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be converted to neoxanthin (N), the last carotenoid product of the β,β-branch. Except for some species (García-Plazaola et al., 2007), L does not undergo β-ring epoxidation and the β,ε-branch thus stops with L, the most abundant carotenoid in leaves.

Each of these carotenoids occupies specific binding sites in the photosynthetic apparatus to fulfill distinct roles. In both PSI and PSII, carotenes (α-C and β-C) are generally bound in core complexes, which also harbor Chl *a* molecules, while the majority of xanthophylls (L, Z, A, V, and N) are bound in light-harvesting antenna complexes together with Chl *a* and Chl *b* molecules (Bassi et al., 1993; Lee and Thornber, 1995). Accumulation of β-C in core complexes is a common feature of diverse photosynthetic organisms, whereas the occurrence of α-C in addition to β-C is restricted to certain taxa. For higher plants, α-C has been found in leaves of some, but not all, shade-tolerant species (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992; Demmig-Adams, 1998; Matsubara et al., 2009). Based on this photoacclimatory behavior, it has been proposed that α-C may function as a light-harvesting pigment while β-C may contribute to photoprotection (Krause et al., 2001), presumably by scavenging singlet oxygen and mediating a cyclic electron transfer around PSII (Tracewell et al., 2001; Telfer, 2005).

Pronounced light-dependent changes are also observed for xanthophyll composition in light-harvesting antenna complexes. In a short term (minutes to hours), operation of the xanthophyll cycle, involving Z, A, and V, modulates levels of Z in a light-dependent manner. It is widely accepted that Z is able to enhance the dissipation of excess light energy from singlet excited Chl while V is not (Demmig-Adams, 1990; Müller et al., 2001). Long-term acclimation (days) to strong irradiance typically results in an increased pool size of the xanthophyll-cycle pigments (V + A + Z) and downsizing of PSII antenna, as indicated by a greater Chl *a*-to-Chl *b* ratio (Demmig-Adams and Adams, 1992; Demmig-Adams, 1998; Matsubara et al., 2009). Based on the observed changes in steady-state amounts of xanthophylls and carotenes following irradiance shifts, alterations in the balance between biosynthesis and degradation, or turnover, have been implicated as a mechanism for long-term adjustment of carotenoid levels in leaves (Förster et al., 2009). However, just how much biosynthesis and degradation of different carotenoids occurs in photosynthesizing green leaves is an open question to date.

In order to gain insight into carotenoid turnover of mature leaves, we conducted $^{14}CO_2$ pulse-chase labeling experiments with Arabidopsis (*Arabidopsis thaliana*) plants. Carotenoid turnover has been studied in algae in the past by applying [$^{14}$C]bicarbonate (Blass et al., 1959; Grumbach et al., 1978); for example, no more than 1% to 2% of the photosynthetically incorporated $^{14}$C was allocated to the lipophilic fraction containing Chl and carotenoid in *Chlorella pyrenoidosa* after a 2-h pulse application (Grumbach et al., 1978). Even lower labeling efficiency is expected for photosynthetic pigments in nongrowing green leaves, in which pigment turnover takes place almost exclusively as part of the maintenance and acclimation of photosynthetic membranes. To overcome this intrinsic but anticipated difficulty, a $^{14}CO_2$ application setup was established for efficient and reproducible $^{14}CO_2$ incorporation in detached leaves of Arabidopsis during a short (30-min) pulse period. Moreover, a method of pigment separation was developed for $^{14}$C detection in concentrated leaf pigment extracts using a radio-HPLC system. Because carotenoid composition exhibits marked sun-shade responses in leaves (Demmig-Adams and Adams, 1992; Demmig-Adams, 1998; Matsubara et al., 2009), $^{14}CO_2$ labeling patterns were studied in three different sets of Arabidopsis plants: (1) plants grown under 130 μmol photons $m^{-2}$ $s^{-1}$ (control light [CL]) and exposed to CL during a chase period of up to 10 h (CL plants); (2) plants acclimated to 1,000 μmol photons $m^{-2}$ $s^{-1}$ (high light [HL]) for 2 weeks and exposed to HL during the chase period (HL plants); and (3) plants grown under CL but exposed to HL during the chase period (CL→HL plants). These treatments simulated short-term (CL→HL) and long-term (CL or HL) responses to irradiance. Finally, as $^{14}$C was found to be rapidly incorporated in β-C and Chl *a* molecules in leaves of wild-type plants, in which β-C represents the only carotene species, $^{14}$C labeling experiments were also conducted with leaves of *lut5* mutants containing both α-C and β-C (Fiore et al., 2006; Kim and DellaPenna, 2006) to compare turnover of the two carotenes.

## RESULTS

### Maximal PSII Efficiency and Carotenoid Composition in Leaves of Wild-Type Plants

Measurements of Chl *a* fluorescence were performed in leaves of wild-type Arabidopsis under CL, HL, or CL→HL conditions (Fig. 1). The maximal PSII efficiency ($F_v/F_m$) remained high in the CL plants when light conditions were not changed, whereas the CL→HL plants showed a significant decrease of $F_v/F_m$ after the transfer to HL at 0 h; the $F_v/F_m$ values of the CL→HL plants decreased rapidly in the first 30 min, then slowly but continuously to values of less than 0.7 after 10 h. The initial rapid decrease accounted for nearly 50% of the PSII impairment measured at 10 h. The HL plants exhibited much less reduction of $F_v/F_m$ during the HL treatment.

Carotenoid compositions were also analyzed (Fig. 2) during the $F_v/F_m$ measurements (Fig. 1) and are expressed relative to Chl *a* (mmol $mol^{-1}$ Chl *a*). The Chl *a* content per unit of leaf area did not alter significantly in different treatments throughout the 10-h experiments (data not shown). In the CL plants, V was the major xanthophyll-cycle pigment that was present besides L, N, and β-C; no Z and only a trace of A could be detected (Fig. 2, A–C). The levels of all

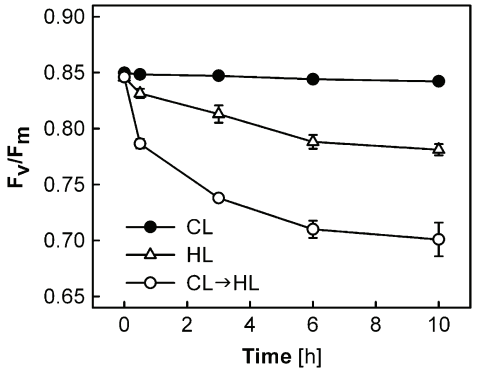


**Figure 1.** $F_v/F_m$ in leaves of Arabidopsis wild-type plants during different light treatments. Plants acclimated and subjected to 130 $\mu$mol photons $m^{-2}$ $s^{-1}$ (CL; black circles), plants acclimated and subjected to 1,000 $\mu$mol photons $m^{-2}$ $s^{-1}$ (HL; white triangles), and plants acclimated to CL and transferred to HL at 0 h (CL→HL; white circles) were used. All data are means ± SE ($n$ = 6). Error bars are shown when they are larger than the symbols.

carotenoid pigments remained unchanged in the CL plants, which also showed no change in $F_v/F_m$ (Fig. 1).

In the CL→HL plants, pigment composition changed considerably during the exposure to HL. In parallel to the rapid decrease of $F_v/F_m$ in the first 30 min (Fig. 1), A + Z were quickly formed at the expense of V via operation of the xanthophyll cycle (Fig. 2D). However, about half of the V pool (approximately 20 mmol $mol^{-1}$ Chl *a*) remained unconverted to A + Z, presumably representing the V molecules that are not accessible to deepoxidase enzymes (Färber et al., 1997). Notably, the levels of A + Z continued to rise, resulting in an increase of the xanthophyll-cycle compounds V + A + Z by a factor of 2.5 after 10 h (Fig. 2D). Also, L increased in the CL→HL plants by approximately 25% by the end of the experiment (Fig. 2F), whereas the contents of N and $\beta$-C stayed constant (Fig. 2E) at the levels of the CL plants (Fig. 2B).

The HL plants had a V + A + Z pool three times greater than that of the CL plants, and no obvious change in V + A + Z was observed during the HL treatment (Fig. 2G). Substantial amounts of A + Z were found initially, but no further deepoxidation occurred during the experiment and V remained the major xanthophyll-cycle pigment in the HL plants. As seen in the CL and CL→HL plants, no change in N or $\beta$-C was measured (Fig. 2H). The L content increased slightly in the first few hours but then returned to the initial level in the second half of the treatment (Fig. 2I).

### $^{14}$C-Labeled Pigments in Leaves of Wild-Type Plants

The increase of V + A + Z and L in the CL→HL plants (Fig. 2, D and F), which started after the maximal light-induced deepoxidation in the xanthophyll cycle, suggests enhanced de novo synthesis of these xanthophylls in response to excess light. In order to examine the synthesis and degradation of carotenoid pigments in mature leaves of Arabidopsis, pulse-chase labeling experiments were conducted with $^{14}CO_2$. After a 30-min pulse of $^{14}CO_2$ under CL, leaves were

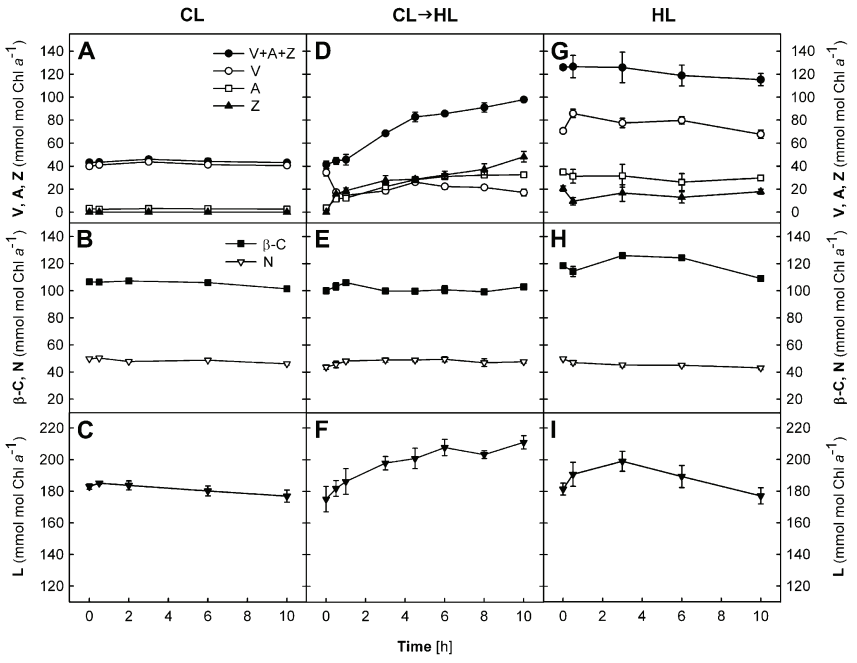


**Figure 2.** Changes in carotenoid contents of wild-type leaves during different light treatments. A to C, CL plants. D to F, CL→HL plants. G to I, HL plants. Carotenoid contents are given relative to the Chl *a* contents (mmol $mol^{-1}$ Chl *a*). Chl contents per unit of leaf area did not change during the experiment for both Chl *a* and Chl *b* (data not shown), with average ratios of Chl *a* to Chl *b* of 3.8 ± 0.03 in the CL and CL→HL plants and 4.77 ± 0.06 in the HL plants. All data are means ± SE ($n$ = 3). Error bars are shown when they are larger than the symbols.

exposed to either CL or HL in ambient air for different durations (chase). The incorporation of $^{14}C$ in photosynthetic pigments was determined by radio-HPLC analysis, and the radioactivity of each pigment was expressed relative to the Chl *a* content.

Chl *a* and *β*-C showed high radioactivity in all samples (Fig. 3, A and B, respectively), but little label was found in Chl *b* or the xanthophylls (see Fig. 7 below). Interestingly, the $^{14}C$ radiolabel of Chl *a* and *β*-C was very similar in the CL and CL→HL plants, suggesting that light conditions during the chase period did not affect the $^{14}C$ levels of these pigments. In the HL plants, significantly higher radioactivity was detected for Chl *a* (150%–190% of the CL plants after 6 h), while somewhat less $^{14}C$ was found in *β*-C (50%–80% of the CL plants). As a result, the $^{14}C$ ratio *β*-C/Chl *a* was much lower in the HL plants (maximum 0.1) than in the CL or CL→HL plants (maximum 0.25; Fig. 3C). The $^{14}C$ distribution between Chl *a* and *β*-C thus seemed to depend on long-term light acclimation states of the leaves.

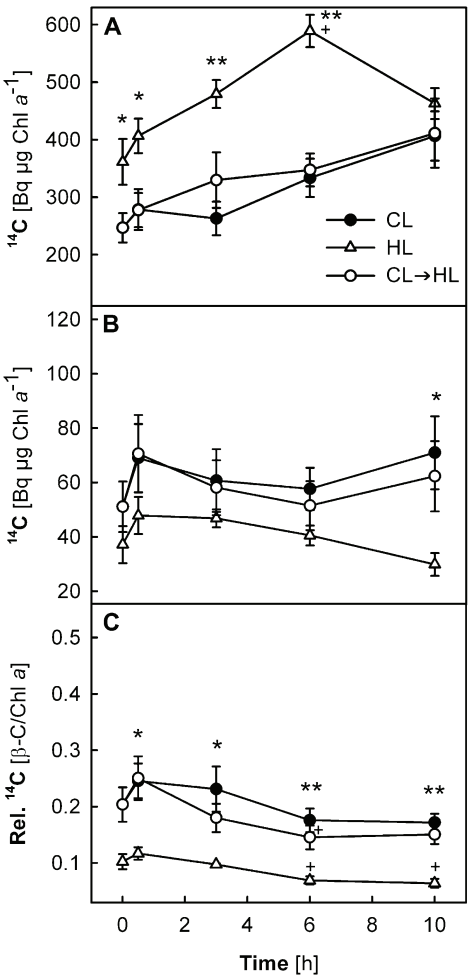


**Figure 3.** A and B, Changes in incorporated $^{14}C$ radioactivity in Chl *a* (A) and *β*-C (B) in leaves of wild-type plants under CL, HL, and CL→HL conditions. C, Ratio between $^{14}C$ radioactivities of the two pigments (*β*-C/Chl *a*). Detached leaves were subjected to $^{14}CO_2$ administration under CL for 30 min (pulse period) and subsequently exposed to either CL or HL for up to 10 h (chase period, starting at 0 h). The $^{14}C$ radioactivities of Chl *a* (A) and *β*-C (B) were normalized to the Chl *a* content measured in the same samples (Bq $\mu g^{-1}$ Chl *a*). Asterisks above the symbols indicate significant differences between the CL and HL plants at each time point; no significant difference was found between data of the CL and CL→HL plants. Plus signs above the symbols show significant differences between the time points within each treatment (i.e. after 3-, 6-, or 10-h chase compared with 0.5 h). ** $P < 0.01$, * and $^{+}$ $P < 0.05$. All data are means ± SE ($n$ = 3–5).

The high $^{14}C$ radiolabeling of Chl *a* and *β*-C just after the pulse suggests a rapid flux of fixed $^{14}C$ into Chl and carotenoid biosynthesis in mature leaves. The differences in $^{14}C$ labeling between the HL and CL plants arose mostly during the 30-min pulse. Afterward, the temporal profiles of $^{14}C$ radioactivity were similar in the CL and HL plants, except for the last time point at 10 h, when radioactivity started to decrease in both Chl *a* and *β*-C in the HL plants. In the CL and CL→HL plants, $^{14}C$ radio signal continued to increase (Chl *a*) or remained nearly unchanged (*β*-C; Fig. 3, A and B). The labeling intensity of *β*-C quickly reached a plateau in all samples already after 0.5 h of chase (Fig. 3B).

### Maximal PSII Efficiency and Carotenoid Composition in Leaves of *lut5* Mutants

The steady-state level of carotene changed little in leaves in the short term (Fig. 2E), whereas radio-HPLC data revealed the dynamic nature of *β*-C homeostasis in photosynthesizing leaves (Fig. 3B). This finding prompted us to examine the incorporation of $^{14}C$ in photosynthetic pigments of the *lut5* mutants, which, as a result of a mutation to a heme-containing *β*-ring hydroxylase gene (Fiore et al., 2006; Kim and DellaPenna, 2006), accumulate a large amount of *α*-C along with a reduced amount of *β*-C in leaves. If the roles of the two carotenes in photosynthesis are not identical, as has been proposed by Krause et al. (2001), they may undergo different turnover during illumination. The *lut5* mutants were grown under CL and exposed to HL as in the CL→HL experiment of the wild-type plants. Similar to the situation in the wild type, $F_v/F_m$ decreased rapidly in the first 30 min of HL, followed by a slow decrease until 10 h (Fig. 4). The initial reduction of $F_v/F_m$ was faster in the *lut5* mutants compared with the wild-type plants (compare with Kim et al., 2009).

The *lut5* mutants are characterized by marked accumulation of *α*-C concomitant with reduced amounts of all other carotenoid pigments (Fiore et al., 2006; Kim and DellaPenna, 2006). Leaves of the CL-grown *lut5* mutants contained approximately 70% V + A + Z, 40% *β*-C, 50% N, and 80% L with respect to the levels in the CL-grown wild-type plants (compare Figs. 2 and 5). The *α*-C level found in the *lut5* mutants (Fig. 5B) was similar to that of *β*-C in the wild-type plants (Fig. 2E).

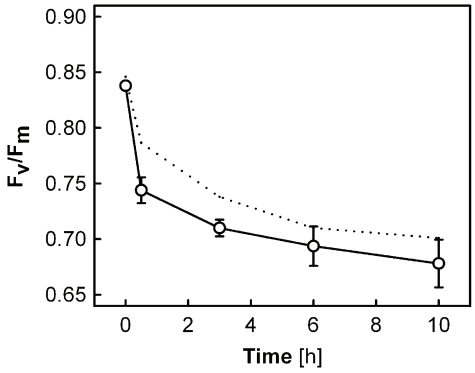


**Figure 4.** $F_v/F_m$ in leaves of *lut5* mutants during HL exposure. Plants grown in CL were transferred to HL at 0 h, as in the CL→HL treatment of the wild-type plants in Figure 1. All data are means ± SE ($n = 6$). For comparison, the data of the wild-type CL→HL plants are also shown (dotted line).

The V + A + Z pool of *lut5* leaves increased continuously from 0.5 to 10 h under HL (Fig. 5A), as observed in leaves of the CL→HL plants of the wild type, but the increase was less pronounced in the *lut5* mutants than in the wild-type plants (by a factor of 2 versus 2.5 in the wild type; Fig. 5A). Concomitantly, a slightly larger increase in L was found in *lut5* (+30% versus +25% in the wild type; Fig. 5C) during the 10-h HL treatment. As was the case for β-C in the wild-type plants (Fig. 2E), neither of the two carotenes in *lut5* showed a significant change in the steady-state level during the experiment (Fig. 5B).

### $^{14}$C-Labeled Pigments in Leaves of *lut5* Mutants

A $^{14}CO_2$ labeling experiment was conducted for the *lut5* mutants using the same pulse-chase protocol as for the CL→HL plants of the wild type (Fig. 3). As seen in the wild-type plants, rapid and strong incorporation of $^{14}$C was found in *lut5* for Chl *a* (Fig. 6A) and carotenes (Fig. 6B), while radioactivity was hardly detectable for Chl *b* and xanthophylls (data not shown). Although the $^{14}$C radioactivity of Chl *a* just after the 30-min pulse (i.e. at 0 h) was somewhat lower in *lut5* than in the wild type, the values reached the wild-type level between 0.5 and 3 h of chase (compare Figs. 3A and 6A).

The $^{14}$C incorporation in β-C was approximately 35% lower in the *lut5* mutants compared with the wild type (compare Figs. 3B and 6B). Nevertheless, taking into account that the β-C content was reduced by 60% in *lut5* (Fig. 5B), relatively more $^{14}$C was incorporated in β-C molecules in *lut5* than in wild-type leaves. Strikingly high $^{14}$C labeling was found for α-C, which is present only in the *lut5* mutants (Fig. 6B). The $^{14}$C radioactivity was up to 60% higher for α-C in *lut5* than for β-C in the wild type (compare Figs. 3B and 6B), in spite of their similar steady-state levels (both approximately 100 mmol mol$^{-1}$ Chl *a*). When α-C and β-C were added together, the radioactivity of carotenes in the *lut5* mutants (Fig. 6B) was more than twice as high as that of β-C in the wild-type plants (Fig. 3B), even though the total amount of carotenes was increased by no more than 40% in *lut5* (Fig. 5B). The $^{14}$C distribution between the two carotenes in *lut5* (Fig. 6B) was nearly proportional to their relative steady-state amounts (Fig. 5B).

The $^{14}$C labeling of α-C and β-C in *lut5* leaves (Fig. 6B) exhibited similar time-course patterns as observed for β-C in wild-type leaves (Fig. 3B). Unlike in the wild type, however, the radioactivity of carotenes started to

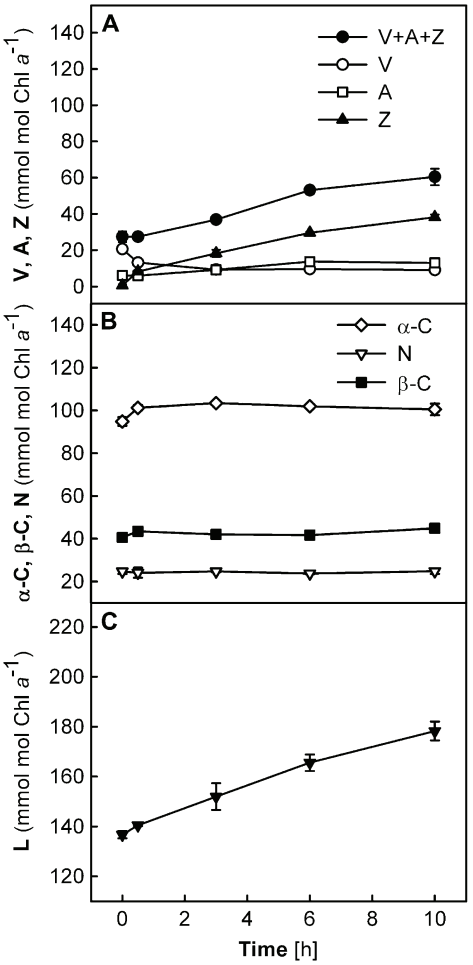


**Figure 5.** Changes in carotenoid contents of *lut5* leaves during HL exposure. Plants grown in CL were transferred to HL at 0 h, as in the CL→HL treatment of the wild-type plants in Figure 2, D to F. Carotenoid contents are given relative to the Chl *a* contents (mmol mol$^{-1}$ Chl *a*). Chl contents per unit of leaf area did not change during the experiment for both Chl *a* and Chl *b* (data not shown), with an average ratio of Chl *a* to Chl *b* of 4.17 ± 0.05. Data are means ± SE ($n = 3$). Error bars are shown when they are larger than the symbols.

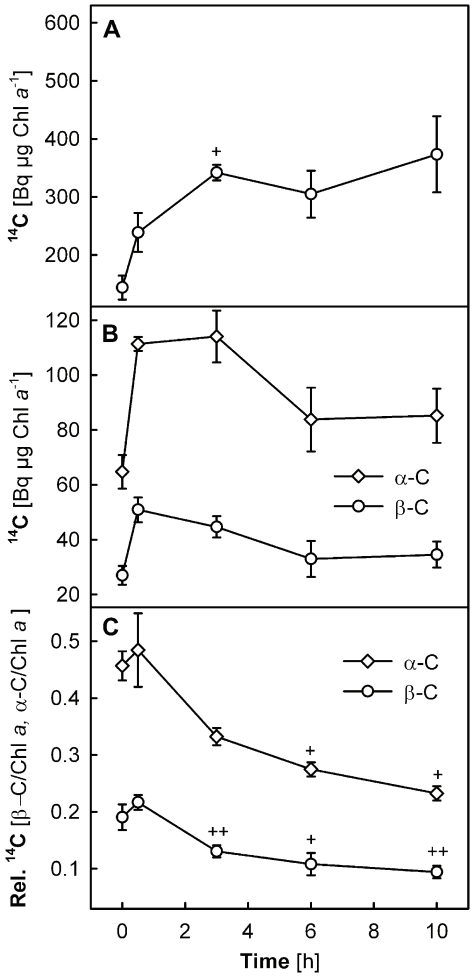


**Figure 6.** A and B, Changes in incorporated $^{14}C$ radioactivity in Chl *a* (A) and α-C and β-C (B) in leaves of *lut5* mutants under CL→HL conditions. C, Ratios between $^{14}C$ radioactivity of carotenes and Chl *a* (β-C/Chl *a* or α-C/Chl *a*). The experimental protocol was as described for the CL→HL treatment of wild-type plants (compare with Fig. 3). The $^{14}C$ radioactivities of Chl *a* or α-C and β-C were normalized to the Chl *a* contents measured in the same samples (Bq $\mu g^{-1}$ Chl *a*). Plus signs above the symbols indicate significant differences between the time points within each treatment (i.e. after 3-, 6-, or 10-h chase compared with 0.5 h). $^{++}$ $P < 0.01$, $^{+}$ $P < 0.05$. All data are means ± SE ($n = 3$).

decrease in *lut5* with increasing duration of the HL exposure. The wild-type level of $^{14}C$ incorporation in Chl *a* (Fig. 6A), together with much greater incorporation in carotenes (Fig. 6B), resulted in remarkably high relative radioactivity of carotenes with respect to that of Chl *a* in *lut5* leaves (Fig. 6C). Notably, the ratio between the $^{14}C$ radioactivities of α-C and Chl *a* approached 0.5 after 30 min of chase. On the other hand, the relative radioactivity of β-C to that of Chl *a* was similar in the *lut5* mutants and the wild-type plants (compare Figs. 3C and 6C). Hence, for a given amount of $^{14}C$ incorporation in Chl *a* molecules, a greater amount of $^{14}C$ was incorporated in carotenes in the *lut5* mutants compared with the wild-type plants grown under the same CL condition.

## DISCUSSION

### Continuous Turnover of Chl *a* and β-C in Photosynthesizing Green Leaves

The $^{14}C$ pulse-chase labeling experiments revealed the dynamic nature of photosynthetic pigment maintenance in mature leaves of Arabidopsis. After the 30-min pulse in CL, Chl *a* and β-C showed rapid incorporation of $^{14}C$ from photosynthetic $CO_2$ fixation (Fig. 3, A and B). Both Chl *a* and β-C are bound in the core complex of PSII (Bassi et al., 1993; Loll et al., 2005), the reaction center of which is susceptible to photoinactivation. The D1 protein of the PSII reaction center undergoes a continuous repair cycle in all light intensities (Tyystjärvi and Aro, 1996) involving PSII disassembly, D1 degradation, insertion of a newly synthesized D1 into the existing PSII, and PSII reassembly (Baena-González and Aro, 2002). Previous studies have shown that translation elongation of mRNA and membrane insertion of new D1 protein depend on the availability of assembly partners (Zhang et al., 1999) and ligation of Chl *a* (Kim et al., 1994; He and Vermaas, 1998) and β-C (Trebst and Depka, 1997). The rapid and concomitant $^{14}C$ labeling of Chl *a* and β-C found in this study (Fig. 3, A and B) demonstrates a continuous flux of newly fixed carbon into Chl *a* and β-C in photosynthesizing leaves, which most likely is important for the PSII repair cycle. The $^{14}C$ labeling of Chl *a* and β-C, but not Chl *b* and xanthophylls bound in light-harvesting antenna complexes (Bassi et al., 1993), is in line with the report by Feierabend and Dehne (1996) for green leaves of rye (*Secale cereale*) seedlings, in which a 4-h incubation with δ-[$^3$H]aminolevulinic acid under illumination resulted in Chl labeling in PSII core complexes but not in light-harvesting antennae and PSI. Chl *b* is synthesized from Chl *a* (or chlorophyllide *a*) by chlorophyllide *a* oxygenase (Tanaka and Tanaka, 2005). When the availability of Chl *b* exceeds the amount needed for import and stabilization of light-harvesting antenna proteins, surplus Chl *b* molecules seem to trigger chlorophyllide *a* oxygenase protein degradation (Yamasato et al., 2005) and thereby suppress Chl *b* biosynthesis. The little $^{14}C$ incorporation in Chl *b* concomitant with strong Chl *a* labeling found in our experiments may indicate such suppression of Chl *b* synthesis in Arabidopsis leaves under CL and HL conditions.

Notably, up to 10 h in HL did not affect the $^{14}C$ signal of Chl *a* and β-C in the CL→HL plants (Fig. 3), suggesting that this short-term exposure to HL did not alter the turnover of these pigments. Consistent with this notion, previous studies documented no change in

D1 protein turnover in leaves of *Brassica napus* and Arabidopsis when irradiance was suddenly increased from growth light to higher light intensities (Sundby et al., 1993; Russell et al., 1995). Accumulation of photoinactivated PSII due to enhanced photodamage, without corresponding up-regulation of repair, could explain the impaired $F_v/F_m$ measured in the CL→HL plants in this study (Fig. 1). In fact, the rate coefficient of PSII repair can decrease under excess light (Lee et al., 2001; He and Chow, 2003), presumably because oxidative conditions inhibit the translation of D1 (Nishiyama et al., 2001).

A different picture emerged for the HL plants. The $F_v/F_m$ decrease in the HL plants was much less pronounced than in the CL→HL plants during the same HL treatment (Fig. 1). Strong $^{14}C$ labeling of Chl *a* recorded in the HL plants throughout the chase period (Fig. 3A), together with the signal decline at 10 h, suggest higher Chl *a* turnover in these plants compared with the CL plants. High-light-induced up-regulation of PSII repair, especially at the step of D1 cleavage by FtsH and Deg proteases (Kato and Sakamoto, 2009), represents one of the vital photoacclimatory responses in leaves (Tyystjärvi et al., 1992; Aro et al., 1994). Furthermore, the ratio of Chl *a* to Chl *b* (see legend to Fig. 2) indicated downsizing of PSII antennae in the HL plants, another predominant acclimatory response in thylakoid membranes under strong light (Anderson et al., 1988). Smaller PSII antennae, and thus the presence of a relatively greater fraction of Chl *a* molecules in the core complexes, may partly account for the higher $^{14}C$ signal of Chl *a* (normalized to the Chl *a* content) in the HL plants. Yet, the difference in labeling intensities of Chl *a* between the CL and HL plants (Fig. 3A) seems too large to be attributed solely to such normalization effects.

The continuous increase of $^{14}C$ in Chl *a* up to 6 or 10 h in leaves of the HL or CL plants, respectively (Fig. 3A), points to an ongoing flux of fixed $^{14}C$ into Chl biosynthesis, or recycling of chlorophyllide and/or phytol moieties from hydrolyzed, $^{14}C$-labeled Chl *a* molecules (Vavilin and Vermaas, 2007). In contrast, the radio signal of β-C reached a peak in all samples after 0.5 h of chase and, what is more, the values were always a little lower in the HL plants than in the CL plants (Fig. 3B). The $^{14}C$ ratio β-C:Chl *a* was no more than 1:10 in the HL plants, or less than half the values of the CL and CL→HL plants (Fig. 3C). These results may imply that the HL plants had higher rates of D1 and Chl *a* turnover, but a lower rate of β-C turnover, than the CL plants. Given the proposed photoprotective functions of β-C in the PSII reaction center (Tracewell et al., 2001; Telfer, 2005), the apparent down-regulation of β-C turnover in the HL plants raises several questions. Does acclimation to strong irradiance lead to less frequent loss of β-C? Do β-C molecules fulfill the same functions in leaves acclimated to contrasting irradiance? The photoacclimatory behavior of β-C turnover in leaves merits further investigation.

**The Sources of the Increases in V + A + Z and L in HL**

Light-induced deepoxidation in the xanthophyll cycle provides Z molecules for the dissipation of excess light energy (Demmig-Adams, 1990) and protection of membrane lipids against photooxidation (Havaux and Niyogi, 1999; Havaux et al., 2004). A large xanthophyll-cycle pool size, which allows rapid formation of many Z molecules upon light exposure, is a common pigment feature of sunlit leaves (Demmig-Adams and Adams, 1992; Demmig-Adams, 1998; Matsubara et al., 2009). The Arabidopsis plants in this study also exhibited a marked increase of V + A + Z during the 2-week acclimation to the HL condition (Fig. 2, A and G). In the short term, HL exposure induced deepoxidation of V to A and Z in the CL→HL plants in the first 30 min, then an increase in V + A + Z to reach 80% of the HL plant level after 10 h (Fig. 2D). This increase in V + A + Z was accompanied by an increase in L by 30 mmol mol$^{-1}$ Chl *a* (Fig. 2F). The involvement of L in energy dissipation has been demonstrated in leaves of higher plant species having the L epoxide cycle (Matsubara et al., 2008) as well as transgenic and mutant plants of Arabidopsis accumulating extra L molecules at the expense of V (Pogson and Rissler, 2000) or in the absence of Z and A (Li et al., 2009). De novo synthesis of both V + A + Z and L was a feature of light acclimation in avocado (*Persea americana*) leaves, and unlike the xanthophyll cycles, relationships with carotenoid precursors were not stoichiometric (Förster et al., 2009). In our study, the total increase in V + A + Z and L in the CL→HL plants during the 10-h HL treatment was 90 mmol mol$^{-1}$ Chl *a*, which suggests the synthesis of these pigments following the maximal deepoxidation in the xanthophyll cycle.

Contrary to our expectation, however, the radio-HPLC analysis did not provide evidence for $^{14}C$ incorporation in xanthophylls in the CL→HL plants throughout the 10-h chase. Although we cannot rule out the possibility that minor radioactivities of xanthophylls were masked by the interfering compounds in our radio-HPLC analysis (Fig. 7), it is unlikely that such weak $^{14}C$ incorporation gave rise to the observed increase in V + A + Z and L. Two scenarios could explain the apparent lack of xanthophyll labeling in our experiments. First, HL-induced de novo synthesis of xanthophylls may have started after the $^{14}C$-labeled precursor pool had been used up and replaced by nonlabeled precursors formed during the chase period under normal air. The fact that the radioactivity of carotenes reached the maximal intensity 0.5 h after the $^{14}C$ pulse labeling (Figs. 3B and 6B) suggests that newly fixed carbon is quickly metabolized and enters the carotenoid biosynthetic pathway in chloroplasts of photosynthesizing leaves. An important implication of this scenario is that short-term high-light exposure enhances de novo synthesis of these xanthophylls without affecting β-C turnover (Fig. 3B), which suggests compartmentalization of xanthophyll and caro-

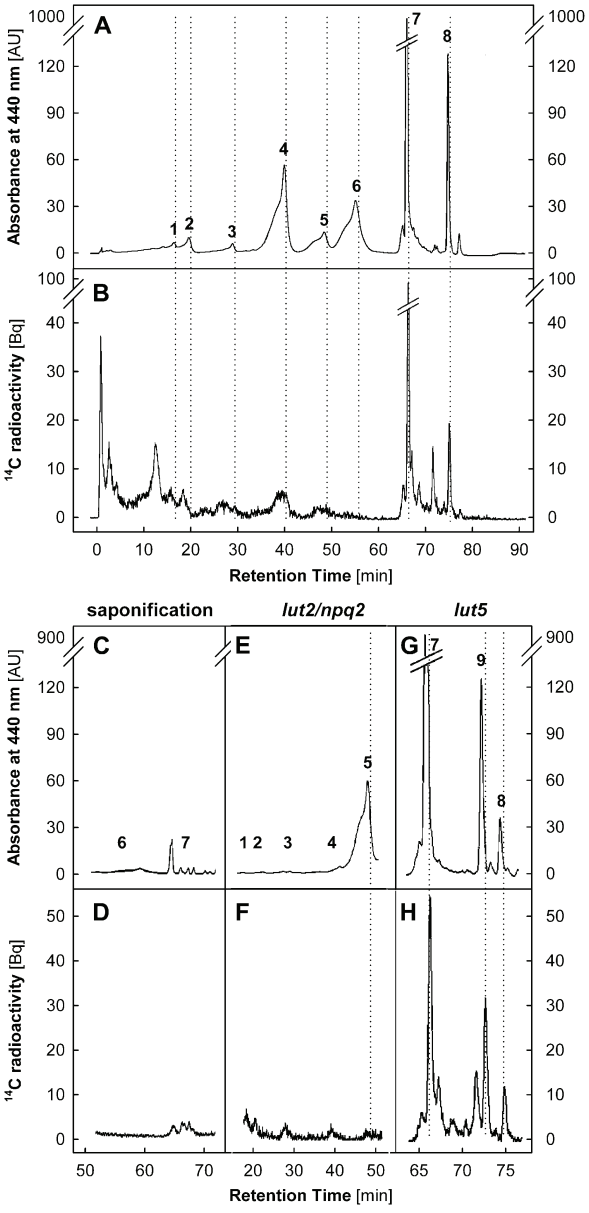


**Figure 7.** Radio-HPLC analysis of photosynthetic pigments. A, A chromatogram (detection at 440 nm) of pigments extracted from a leaf of wild-type Arabidopsis grown in CL and exposed to HL for 3 h. Peak 1, V; peak 2, N; peak 3, A; peak 4, L; peak 5, Z; peak 6, Chl *b*; peak 7, Chl *a*; peak 8, β-C. AU, Arbitrary units. B, Simultaneous radiogram of $^{14}$C-labeled compounds. Dotted lines indicate the expected positions of pigment peaks in the radiogram, with a 20-s offset compared with the corresponding peaks in the chromatogram due to the sequential detection by the UV-visible light and radio detectors. Radioactivity was hardly detectable at peak positions 2, 5, and 6. C and D, Saponification eliminated Chl peaks 6 and 7 in the chromatogram (C) as well as the prominent peak 7 in the corresponding radiogram (D). E and F, Lack of xanthophyll pigments at peaks 1 to 4 concomitant with pronounced accumulation of Z at peak 5 in *lut2*/*npq2* mutants (E) did not eliminate wild-type levels of radiolabeling at less than 40 min (F). G and H, Appearance of peak 9 (α-C) at the expense of peak 8 (β-C) in *lut5* mutants (G) was accompanied by the appearance of a new peak in the radiogram at the expected position of α-C together with a smaller peak of β-C (H).

tene biosynthesis. Notably, a recent study in Arabidopsis has shown that an irradiance increase from 150 to 1,000 μmol photons $m^{-2}$ $s^{-1}$ rapidly induces (within 10–60 min) transcriptional up-regulation of a gene encoding nonheme diiron β-ring hydroxylase (Cuttriss et al., 2007), which catalyzes the hydroxylation of β-C to Z (Kim et al., 2009). Pulse-chase labeling with $^{14}CO_2$ not before but during the HL-induced accumulation of V + A + Z and L could test if de novo biosynthesis of these xanthophylls is enhanced after transfer to strong light.

Additionally, one could postulate the existence of xanthophyll precursor pools that are not directly linked with photosynthetic $CO_2$ fixation. A potential candidate for such a pool is carotene molecules in PSII core complexes. It has been proposed for *Chlamydomonas reinhardtii* that hydroxylation of β-C, which is released from the PSII reaction center during D1 turnover, may contribute to Z formation under strong light, along with deepoxidation of V to Z in the xanthophyll cycle (Depka et al., 1998). Since β-C molecules are bound not only in the reaction center but also in peripheral regions of the core antenna complexes CP47 and CP43 (Loll et al., 2005), it is possible that some of these β-C molecules in the PSII core complexes dissociate from apoproteins during disassembly and become available for hydroxylation. According to this hypothesis, preexisting, nonlabeled carotene molecules in PSII core complexes may have served as a precursor pool for the additional xanthophylls in our experiments. Although possible, this cannot be the only mechanism of the HL-induced xanthophyll accumulation in leaves of Arabidopsis. While the accumulation of L was slightly enhanced in *lut5* leaves containing α-C (Fig. 5, B and C), which is the precursor of L, it also happened in the wild-type plants having no α-C (Fig. 2, E and F). If we attribute the HL-induced increase of V + A + Z in the CL→HL plants (approximately 60 mmol $mol^{-1}$ Chl *a* by 10 h; Fig. 2D) solely to β-C from the PSII repair cycle, it follows that nearly 60% of the steady-state amount of β-C (Fig. 2E) must have been hydroxylated during the 10-h HL exposure. Assuming a PSII:PSI ratio of 1.5 to 1.7 (Fan et al., 2007) and 11 β-C and 100 Chl *a* molecules for monomeric PSII (with two trimeric major light-harvesting antenna complexes) and 26 β-C and 160 Chl *a* molecules for PSI (Bassi et al., 1993; Ballottari et al., 2004; Liu et al., 2004; Loll et al., 2005), the observed V + A + Z increase would correspond to, for instance, one photoinactivation-repair cycle of the entire PSII population and thereby hydroxylation of all the nonlabeled (and nonbleached) β-C from PSII, including nine β-C molecules in CP47 and CP43. If the two β-C molecules in the PSII reaction center are the

only substrates for hydroxylase during D1 turnover, five photoinactivation-repair cycles of the entire PSII population would be needed. Although such high rates of D1 turnover may happen in leaves under strong irradiance (Chow et al., 2002), we then would expect to see some radioactivity appearing at Z during the 10-h chase due to hydroxylation of $^{14}$C-labeled $\beta$-C molecules. This was not the case.

Whatever the mechanism(s) and precursor pool(s) for HL-induced formation of additional xanthophylls, an important question arises as to the fate of carotenes in PSII under nonstressful conditions, as in the CL plants. Note that PSII photoinactivation and D1 turnover (Tyystjärvi and Aro, 1996; Lee et al., 2001) as well as Chl and carotene biosynthesis (Fig. 3) continuously take place in the light, while steady-state levels of these molecules do not change in the short term (Fig. 2, B, E, and H). If carotenes in photoinactivated PSII are neither bleached (Tracewell et al., 2001; Telfer 2005) nor hydroxylated during the repair cycle (Depka et al., 1998) under nonstressful irradiance, they may be degraded by carotenoid cleavage dioxygenase enzymes (CCD; Auldridge et al., 2006b). It has been shown that the expression level of certain CCDs can influence the carotenoid turnover rate in seeds, fruits, flower petals, and roots (Auldridge et al., 2006a, 2006b; Ohmiya et al., 2006; García-Limones et al., 2008). For chloroplasts of green leaves, on the other hand, not much is known about the occurrence and pathway of carotenoid catabolism, with the exception of abscisic acid biosynthesis (Milborrow, 2001). Plants lacking CCDs could offer an interesting system to examine carotenoid degradation in chloroplasts and leaves.

### Different Turnover of $\alpha$-C and $\beta$-C

The lack of the *lut5* gene product, a heme-containing cytochrome P450 monooxygenase that catalyzes $\beta$-ring hydroxylation of $\alpha$-C, results in the accumulation of $\alpha$-C in PSII and PSI as well as reduced levels of all other carotenoids in leaves of Arabidopsis (Fiore et al., 2006; Kim and DellaPenna, 2006). Physiologically, leaves of the *lut5* mutants have a smaller capacity of light-induced thermal energy dissipation compared with the wild-type plants (Dall'Osto et al., 2007; Kim et al., 2009) and show more rapid decrease of $F_v/F_m$ when exposed to strong light. While these changes do not cause severe photoinhibition and photodamage under moderately high light (750 or 1,000 $\mu$mol photons $m^{-2}$ $s^{-1}$; Dall'Osto et al., 2007; Kim et al., 2009), leaves of the *lut5* mutants bleach after transfer to higher irradiance (1,800 $\mu$mol photons $m^{-2}$ $s^{-1}$; Kim et al., 2009).

In this study, the $F_v/F_m$ decrease following the transfer from CL to HL (from 130–1,000 $\mu$mol photons $m^{-2}$ $s^{-1}$) was not so pronounced in *lut5* leaves, although it happened faster than in the wild type (Fig. 4). Under this "tolerable" HL condition, the $^{14}CO_2$ pulse-chase labeling experiment revealed strikingly high $^{14}$C incorporation in carotenes of *lut5* leaves. Enhanced radioactivity of carotenes (especially $\alpha$-C) was observed initially when the leaves were still under CL (Fig. 6B), indicating high carotene turnover even under nonstressful environments. Importantly, as the labeling of Chl *a* was comparable in the two genotypes (compare Figs. 3A and 6A), the *lut5* mutation seems to specifically increase carotene turnover, but not Chl *a* turnover. Judging by the lack of $^{14}$C incorporation in L and Z, high carotene turnover in *lut5* did not apparently result in enhanced hydroxylation of $^{14}$C-labeled $\alpha$-C and $\beta$-C to L and Z, respectively. It can be hypothesized that $\alpha$-C is more prone to degradation by photooxidation (Tracewell et al., 2001; Telfer, 2005) or enzymatic cleavage (Auldridge et al., 2006) than $\beta$-C. High degradation and turnover rates of carotenes, in addition to the smaller capacity of photoprotective light energy dissipation (Dall'Osto et al., 2007; Kim et al., 2009), may contribute to the high-light sensitivity of the *lut5* mutants.

Natural accumulation of $\alpha$-C is found mainly in leaves under shaded or deeply shaded environments (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992; Demmig-Adams, 1998; Matsubara et al., 2009). A recent large pigment survey of tropical forest species, commonly containing $\alpha$-C in leaves, indicated a selection pressure that generally suppresses $\alpha$-C accumulation in sun-exposed conditions (Matsubara et al., 2009). Since the balance between the two carotenes shifts from $\alpha$-C to $\beta$-C with increasing growth irradiance (Thayer and Björkman, 1990; Matsubara et al., 2009), it has been proposed that $\beta$-C may contribute to efficient photoprotection whereas $\alpha$-C may function as light-harvesting pigment (Krause et al., 2001). Our results from the *lut5* mutants, which demonstrate different turnover of $\alpha$-C and $\beta$-C during the PSII repair cycle, are consistent with this notion and underscore the interface between PSII photoprotection/photoinactivation and the regulation of carotenoid biosynthesis during photoacclimation.

## CONCLUSION

The $^{14}CO_2$ pulse-chase labeling experiments revealed "hidden" phenotypes of pigment turnover (Chl *a*, $\alpha$-C, and $\beta$-C) in mature, photosynthesizing leaves of Arabidopsis. Despite their constant steady-state levels, these pigments undergo continuous turnover even under nonstressful light conditions. Turnover rates of Chl *a* and carotenes seem to be influenced by photoacclimation states of leaves and by the carotene species. On the other hand, de novo synthesis of xanthophylls (V + A + Z and L) could not be demonstrated by the pulse-chase protocol used for $^{14}CO_2$ labeling, even though steady-state amounts of these pigments substantially increased in response to high-light stress during the chase, which very likely involves xanthophyll biosynthesis. Most importantly, our results emphasize the need to consider carotenoid biosynthesis in the course of photoprotection

in relation to pigment dynamics during the D1 repair cycle following PSII photoinactivation. Together, these findings invite further investigation into the regulatory mechanisms of synthesis and degradation of photosynthetic pigments, which are essential for the maintenance and acclimation of photosynthetic membranes in leaves.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 wild type and *lut2/npq2* and *lut5* mutants (mutant seeds kindly provided by Roberto Bassi, University of Verona) were grown in soil (ED 73 Einheitserde; Balster Einheitserdewerk) in a growth cabinet with a 12-h/12-h day/night photoperiod under a constant relative air humidity of 50% and at 22°C/18°C (day/night) air temperature. Photosynthetically active photon flux density of approximately 130 $\mu$mol photons $m^{-2}$ $s^{-1}$ (CL) was provided by a combination of FLUORA and warm white fluorescent tubes (Osram). At the beginning and at the end of the day period, the light intensity in the growth cabinet was gradually increased or decreased over 1 h to simulate sunrise and sunset. After germination and 3 weeks of cultivation under the above CL condition, some plants were transferred to HL condition (1,000 $\mu$mol photons $m^{-2}$ $s^{-1}$) provided by Master HPI-T Plus lamps (Philips) without changing the photoperiod and the relative air humidity but at a constant air temperature of 19°C. These plants were acclimated to the HL condition for about 2 weeks before experiments. Mature leaves (up to four leaves per plant) of 5- to 7-week-old plants were used for all experiments.

### Light Treatments and Chl *a* Fluorescence Measurements

A set of plants grown under the CL condition was exposed to HL (CL→HL plants) in the growth chamber, starting after the sunrise period. For comparison, another set of the CL-grown plants were kept under the CL condition (CL plants). All HL-grown plants were subjected to the HL condition (HL plants). After different durations of the CL→HL, CL, or HL treatment, mature leaves were detached from the plants, placed on a moist tissue, and dark adapted for 15 min using leaf clips. $F_v/F_m$ (fluorescence nomenclature according to van Kooten and Snel, 1990) was determined by measuring Chl *a* fluorescence in the dark-adapted leaves with a Handy PEA (Hansatech).

### Analysis of Photosynthetic Pigments

For pigment analysis, leaf discs (1.13 $cm^2$) were taken directly from attached leaves during the light treatments without dark adaptation. The discs were frozen in liquid nitrogen and stored at −20°C for up to 2 weeks until acetone extraction. Pigment extraction and the HPLC analysis were performed according to the method described by Matsubara et al. (2005) using an Allsphere ODS-1 C18 column (5 $\mu$m, 250 × 4.6 mm; Alltech) and a corresponding guard column (5 $\mu$m, 7.5 × 4.6 mm; Alltech). Pigments were detected by a Waters PAD-996 UV/visible light detector (Waters), and peak areas were integrated at 440 nm with Waters Empower software. Contents of different carotenoids were expressed relative to the Chl *a* amount (mmol $mol^{-1}$ Chl *a*) for each sample.

### Isotope Labeling with $^{14}CO_2$

Labeling with $^{14}CO_2$ was performed in a gas-circuit system including a valve for opening or closing the system, a reaction vessel, a gas pump (1.3 L $min^{-1}$ flow rate), a dew-point trap (kept at 5°C–10°C), and a leaf chamber (12 $cm^3$); the total volume of the system was approximately 20 $cm^3$. Aqueous sodium [$^{14}$C]carbonate solution (7.4 MBq; GE Healthcare) was injected into 20 $\mu$L of perchloric acid (70%) and heated to 90°C in the sealed reaction vessel, ensuring fast liberation of $^{14}CO_2$. After 25 min of application, remaining $^{14}CO_2$ in the system was absorbed by soda lime granules (Carbosorb; BDH Laboratory Supplies) for 5 min before opening the leaf chamber.

Four detached leaves were placed side by side in the sealed leaf chamber and supplied with water through the petioles from cavities of the chamber bottom. After the 1-h "sunrise" period (see above), $^{14}CO_2$ administration started under the CL condition at 19°C. Immediately after $^{14}CO_2$ labeling, the leaves were floated on water in a petri dish with the adaxial side facing ambient air and, during this chase period, were subjected to either the CL or HL in the growth chamber, corresponding to the CL→HL, CL, and HL treatments of the fluorescence experiment described above. Whole lamina of $^{14}CO_2$-labeled leaves were sampled at different times of the chase and frozen in liquid nitrogen for radio-HPLC analysis.

### Radio-HPLC Analysis of $^{14}$C-Labeled Pigments

Pigments were extracted from whole lamina first with 1.2 mL of acetone, followed by two-phase extraction (ethyl acetate and water) twice according to the method of Pogson et al. (1996). The extracts were concentrated to a final volume of 500 $\mu$L under a nitrogen gas stream and dim laboratory light. The concentrated extracts were either immediately analyzed by radio-HPLC or stored at −20°C for less than 5 h until analysis.

Analytical HPLC was carried out by a PU-1850 HPLC system (Jasco) equipped with an autosampler (Gynkotek) and a UV/visible light detector (Jasco), followed by a radio monitor (radioflow detector LB 509) with a solid yttrium-gadolinium scintillator cell (YG-150-S-4; both from Berthold Technologies). This setup for sequential detection with a UV/visible light detector and a radio detector resulted in a constant offset of approximately 20 s (Fig. 7, A and B).

In order to obtain good signal-to-noise ratios in the radioactivity detection, 100 $\mu$L of concentrated leaf extracts was injected into the HPLC system. An HPLC method using a Prontosil reverse-phase C30 column (3 $\mu$m, 250 × 4.6 mm; Bischoff) and a corresponding guard column (3 $\mu$m, 10 × 4.0 mm; Bischoff) was established for the radio-HPLC analysis; the amounts of pigments per injection were too large for separation with the HPLC method based on the C18 column described above. Elution was carried out at room temperature and a flow rate of 1.2 mL $min^{-1}$ with methanol:water (87:13 [v/v]; solvent A) and 100% tert-butyl methyl ether (solvent B) according to the following program: isocratic at 85:15 for 19 min, followed by a linear gradient to 80:20 in 1 min, then isocratic at 80:20 for 38 min, a linear gradient to 40:60 in 3 min, isocratic at 40:60 for 17 min, and finally a linear gradient back to 85:15 in 3 min. The column was equilibrated for 20 min before each injection.

Peak integration was performed with RadioStar software (Berthold Technologies) for both UV/visible light chromatograms (440 nm) and radiograms. Peak areas of the radiogram were normalized to the Chl *a* content given by the corresponding 440-nm chromatogram and expressed as Bq $\mu g^{-1}$ Chl *a*.

### Identification of Photosynthetic Pigments in the Radiograms

Chl peaks were confirmed by saponification of acetone extracts (1.2 mL) with 0.5 g of Ambersep 900 OH (Sigma-Aldrich) on ice for 20 min, as described by Larsen and Christensen (2005). Saponification efficiently removed Chl *a* and Chl *b* from the extract (Fig. 7C), resulting in disappearance of the Chl *a* peak in the radiogram (Fig. 7D). No radio signal was detected for Chl *b* in any samples. The saponification procedure did not affect carotenoid contents (data not shown).

The $^{14}CO_2$ labeling was also conducted for Arabidopsis carotenoid mutants *lut2/npq2* and *lut5*. In the *lut2/npq2* mutants (Havaux et al., 2004), constitutively containing a large amount of Z but lacking all other xanthophylls (V, A, N, and L; Fig. 7E), the radiogram revealed interfering lipophilic compounds in the region where these xanthophylls are expected (Fig. 7F). No radio peak of Z appeared, despite the strong accumulation of this pigment in the *lut2/npq2* mutants. In the *lut5* mutants, which accumulate $\alpha$-C at the expense of $\beta$-C (Fig. 7G; Fiore et al., 2006, Kim and DellaPenna, 2006), a new peak was observed in the radiogram at the expected position of $\alpha$-C together with a smaller peak of $\beta$-C (Fig. 7H).

### Statistical Data Analysis

$^{14}$C labeling data were statistically tested by *t* test. For the experiments with the wild-type plants, differences between the CL and HL plants or the CL and CL→HL plants were tested for statistical significance at each sampling time point. Additionally, statistical significance was also checked for time-course variations within each treatment by comparing data after 3, 6, and 10 h of chase with 0.5 h. The values at 0.5 h were chosen as reference since the $^{14}$C

intensity of carotenes reached the maximal levels at this time point. Time-course changes in the experiment with the *lut5* mutants were also statistically tested.

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## 6.3 Altered Turnover of β-Carotene and Chlorophyll *a* in Arabidopsis Leaves Treated with Lincomycin or Norflurazon (Beisel et al., 2010 submitted)

**TITLE:**

**Altered Turnover of β-Carotene and Chlorophyll *a* in Arabidopsis Leaves Treated with Lincomycin or Norflurazon**

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**ABSTRACT**

Interactions between β-carotene (β-C) and chlorophyll *a* (Chl *a*) turnover were investigated in relation to photoinhibition and D1 protein turnover in mature leaves of Arabidopsis (*Arabidopsis thaliana*) by $^{14}CO_2$ pulse-chase labeling. Following a 2-h treatment of leaves with water, lincomycin (Linco; inhibitor of D1 protein synthesis) or norflurazon (NF; inhibitor of carotenoid biosynthesis at phytoene desaturase) in dark, $^{14}CO_2$ was applied to the leaves for 30 min under control light (CL; 130 µmol photons $m^{-2}$ $s^{-1}$) conditions, followed by exposure to either CL or high light (CL→HL; 1000 µmol photons $m^{-2}$ $s^{-1}$) in ambient $CO_2$ for up to 6 h. Under both light conditions, $^{14}C$ incorporation was strongly decreased in Chl *a* and slightly less in β-C for Linco-treated leaves which showed a marked decline of Fv/Fm and β-C content compared to water-treated leaves. Partial inhibition of carotenoid biosynthesis by NF treatment caused no or only a minor decrease of Fv/Fm and Chl *a* turnover under CL and CL→HL conditions whereas a significant reduction of β-C content was observed. Extremely high $^{14}C$ labeling was found in NF-treated leaves for phytoene, the substrate of phytoene desaturase, with radioactivity much higher than the highest values found for β-C. The results suggest a coordinated turnover of Chl *a* and D1 protein but somewhat different regulation for β-C turnover in Arabidopsis leaves. Inhibition of carotenoid biosynthesis by NF seems to initially enhance metabolic flux in the pathway upstream of phytoene, presumably compensating for short supply of β-C.

**INTRODUCTION**

Light is essential for photosynthesis but it can also cause oxidative damage to the photosynthetic apparatus when the light energy absorbed by chlorophyll *a* (Chl *a*) molecules cannot be utilized for photochemical reactions. A range of photoprotective mechanisms exist in and around pigment-protein complexes of photosynthetic membranes (thylakoids) to minimize photooxidative damage resulting from formation of triplet-state Chl and singlet $O_2$ (Demmig-Adams, 1990; Noctor and Foyer, 1998; Müller et al., 2001; Maeda and DellaPenna 2007). Nevertheless, photosystem II (PSII), the site of water oxidation and plastoquinone reduction, undergoes light-induced inactivation even under low irradiance, which manifests itself as irreversible damage to the reaction center polypeptide D1 (Tyystjärvi and Aro, 1996; Keren et al., 1997). Damaged D1 protein of photoinactivated PSII is replaced by a new copy through an elaborate repair cycle operating between stacked grana and non-stacked stroma regions of thylakoids (Melis, 1999; Baena-González and Aro, 2002). The D1 and D2 polypeptides of PSII reaction center bind six Chl *a*, two pheophytin, two plastoquinone and two β-carotene (β-C) molecules (Kobayashi et al., 1990). Whereas Chl *a*, pheophytin and plastoquinone are engaged in PSII electron transfer as redox-active cofactors, β-C is thought to play a photoprotective role (Telfer, 2005; Tracewell et al., 2001). It has been suggested that translation elongation and membrane insertion of new D1 protein during the D1 repair cycle requires assembly partners (Zhang et al., 1999) as well as ligation of Chl *a* (Kim et al., 1994; He and Vermaas, 1998) and β-C (Trebst and Depka, 1997). Whenever the repair and turnover of D1 cannot keep pace with the rate of photodamage, e.g. upon irradiance increase or under stress conditions, the overall quantum yield of PSII declines ('photoinhibition').

In parallel with the continuous D1 turnover in PSII reaction center in the light, interconversions between epoxidized and de-epoxidized xanthophylls (xanthophyll cycle) take place in thylakoids, catalyzed by two enzymes violaxanthin (V) de-epoxidase and zeaxanthin (Z) epoxidase (Jahns et al., 2009). Light-induced acidification of thylakoid lumen activates V de-epoxidase which converts V molecules released from light-harvesting antenna complexes into antheraxanthin (A) and Z. The reactions are reversed by the activity of Z epoxidase, which becomes evident when V de-epoxidase is inactive in the dark or under non-stressful light intensities. Accumulation of Z via operation of the xanthophyll cycle enhances non-photochemical quenching of singlet-excited Chl (Demmig-Adams, 1990; Müller et al., 2001) and provides antioxidative protection (Havaux and Niyogi, 1999) under strong illumination. Corresponding to these photoprotective functions, sunlit leaves typically contain larger amounts of the xanthophyll-cycle pigments (V+A+Z) than leaves in shaded environments (Demmig-Adams and Adams, 1992; Matsubara et al., 2009).

De novo synthesis of V+A+Z (especially Z) as well as β-C and/or lutein (L) has been observed in leaves in response to high-light exposure and associated with upregulation of

carotenoid biosynthesis to enhance photoprotection (García-Plazaola et al., 2002; Förster et al., 2009). Recently, continuous turnover of β-C and Chl *a* has been demonstrated in Arabidopsis (*Arabidopsis thaliana*) leaves by $^{14}CO_2$ pulse-chase labeling (Beisel et al., 2010). Based on rapid $^{14}C$ incorporation in these PSII core-complex pigments, but not in light-harvesting antenna pigments (xanthophylls and Chl *b*), turnover of β-C and Chl *a* observed under both stressful and non-stressful light conditions was considered as part of the D1 repair cycle. Since Z is synthesized by β-ring hydroxylation of β-C, a direct link between β-C turnover during the D1 repair cycle and stress-induced Z accumulation has been proposed for *Chlamydomonas reinhardtii* (Depka et al., 1998). An important implication of these previous observations is that carotenoid metabolism in chloroplasts can sense and respond to environmental changes which alter the photoprotective demand for different carotenoids. In support of this notion, increased transcript abundance of β-ring hydroxylase genes, the products of which predominantly catalyze hydroxylation of β-C to Z (Kim et al., 2009), has been reported for Arabidopsis leaves shortly (~1 h) after high-light exposure (Rossel et al., 2002; Cuttriss et al., 2007).

If degradation and synthesis of β-C and Chl *a* play a part in the D1 repair cycle in leaves, their turnover may be regulated in conjunction with concurrent turnover of D1 protein. In order to gain insights into interactions between turnover of β-C, Chl *a* and D1, we have conducted $^{14}CO_2$ pulse-chase labeling experiments in Arabidopsis leaves following treatments with inhibitors of D1 or β-C synthesis. Lincomycin (Linco), an inhibitor of chloroplast protein synthesis, arrests translation of plastid-encoded *psbA* gene product (D1), and thus repair of photoinactivated PSII, resulting in a reduced PSII efficiency in leaves (Lee et al., 2001; Tyystjärvi and Aro, 1996). Synthesis of β-C can be inhibited by norflurazon (NF) which impairs the catalytic activity of phytoene (Phy) desaturase upstream of β-C. Carotenoid (β-C) depletion by NF incubation diminishes D1 as well as photosynthetic activity in green algae (Sandmann et al., 1993; Trebst and Depka, 1997). Also in higher plants, NF treatment leads to impaired PSII efficiency, decreased contents of D1 and other Chl-binding protein complexes of PSII, and ultimately, bleaching (Markgraf and Oelmüller, 1991; Corona et al., 1996; Xu et al., 2000; Welsch et al., 2003). After pre-treatment with these inhibitors and a chase period of up to 6 h under different light conditions, pigments were extracted from leaves and radioactivity of each $^{14}C$-labeled pigment was measured. The results are discussed in terms of whether the turnover processes of β-C, Chl *a* and D1 are coupled with each other in mature Arabidopsis leaves, and further, how carotenoid synthesis reacts on NF-induced restriction of metabolic flux in the pathway under stressful and non-stressful light environments.

## RESULTS

### Maximal PSII Efficiency

After detaching leaves from Arabidopsis plants grown under 130 µmol photons $m^{-2}$ $s^{-1}$ (control light, CL), petioles were immediately put in water ($H_2O$), 3 mM Linco or 70 µM NF solution and the leaves were kept for 2 h in the dark with an airflow forcing transpiration. The leaves were then floated on water or diluted inhibitor solutions (1 mM Linco or 10 µM NF) with the adaxial surface facing the air, and placed under 130 µmol photons $m^{-2}$ $s^{-1}$ (CL treatment) or 1000 µmol photons $m^{-2}$ $s^{-1}$ (CL→HL treatment) starting at 0 h (Fig. 1). Measurements of Chl *a* fluorescence were performed at different time points during the light treatments. The maximal PSII efficiency (Fv/Fm) remained high in $H_2O$-treated and NF-treated leaves under CL, whereas the corresponding leaves in CL→HL showed a significant decrease (Fig. 1A and C). While the initial (up to 0.5 h) rapid decrease under CL→HL was similar in $H_2O$-treated and NF-treated leaves, the subsequent slow decrease of Fv/Fm was more pronounced in NF-treated leaves. The leaves treated with Linco exhibited a significant and almost linear reduction of Fv/Fm from 0.84 to 0.62 over the 6 h under CL (Fig. 1B), whereas Fv/Fm declined rapidly to less than 0.6 in the first 30 min and to almost 0 after 6 h under CL→HL conditions.

### Chlorophyll and Carotenoid Composition

Chlorophyll and carotenoid compositions were analyzed in leaves during the Fv/Fm measurements under CL (Fig. 2) and CL→HL conditions (Fig. 3). In both conditions, neither Chl *a* nor Chl *b* content of leaves changed significantly in the three treatments throughout the 6-h experiment (Figs. 2A, D, G and 3A, D, G).

Under CL, V was generally the major xanthophyll-cycle pigment in leaves (Fig. 2B, E, H); only traces of A and Z could be detected. Except for a small and transient decrease of V in $H_2O$-treated leaves shortly after the dark-to-CL transfer at 0 h, the levels of the xanthophyll-cycle pigments remained practically unchanged during the experiment and the values were comparable between the leaves treated with $H_2O$, Linco and NF. The initial levels of L, N and β-C were also similar in the three treatments (Fig. 2C, F, I). However, while L and N remained nearly constant, the β-C contents significantly decreased by 6 h in Linco-treated leaves and by 3 h in NF-treated leaves.

The carotenoid composition changed substantially in the leaves of all three treatments under CL→HL conditions (Fig. 3). In the first 30 min, $H_2O$-treated leaves quickly accumulated Z and some A at the expense of V via operation of the xanthophyll cycle (Fig. 3B); the de-epoxidation state (DPS), calculated as (A+Z)/(V+A+Z), was 0.7 at 0.5 h. The highest DPS values (0.9) were reached at 3 h, concomitant with a significant increase (+50% compared with 0 h) in the total amount of the xanthophyll-cycle pigments (V+A+Z). Thereafter, no

further change in DPS or V+A+Z was observed. Lincomycin-treated leaves showed similar but slower changes compared with $H_2O$-treated leaves (Fig. 3E); DPS increased more gradually after the first 30 min (0.65, 0.75 and 0.85 after 0.5 h, 3 h and 6 h, respectively) and V+A+Z increased by 30% between 3 h and 6 h. The operation of the xanthophyll cycle was also evident in NF-treated leaves under CL→HL (Fig. 3H). Although the DPS values of NF-treated leaves were very similar to those of $H_2O$-treated leaves, the increase in V+A+Z found in these leaves between 0.5 h and 3 h was only minor and statistically not significant.

Regardless of the inhibitor treatments, N remained unchanged under CL→HL conditions (Fig. 3C, F, I). The variations in L were mostly not significant, with the exception of the somewhat higher values (+15% compared with 0 h) in $H_2O$-treated leaves at 3 h and 6 h (Fig. 3C). This increase in L coincided with the increase in V+A+Z in these leaves (Fig. 3B). As seen under CL, the β-C contents were stable in $H_2O$-treated leaves under CL→HL (Fig. 3C) whereas Linco-treated and NF-treated leaves both showed a significant decrease (Fig. 3F and I). The levels of β-C decreased more rapidly and/or strongly in CL→HL than in CL, with a total reduction under CL→HL after 6 h being -32% and -27% in Linco-treated and NF-treated leaves, respectively, compared with -6% and -12% in the corresponding leaves under CL.

**$^{14}$C-Labeled Pigments**

Pulse-chase labeling experiments were conducted with $^{14}CO_2$ in order to examine synthesis and degradation of photosynthetic pigments in Arabidopsis leaves in the presence of Linco or NF. Following the treatment with either $H_2O$ or the inhibitors (3 mM Linco or 70 µM NF) for 2 h in the dark, leaves were subjected to a 30-min pulse application of $^{14}CO_2$ under CL, with their petioles incubated in the corresponding solutions ($H_2O$, 1 mM Linco or 10 µM NF). Then, leaves were placed under CL or CL→HL conditions in the ambient air for different duration (chase) while floating on $H_2O$, Linco (1 mM) or NF (10 µM) solution. Incorporation of $^{14}$C in photosynthetic pigments was determined by radio-HPLC analysis (Beisel et al., 2010), and the radioactivity of each pigment was expressed relative to the Chl *a* content.

For all treatments, rapid and strong incorporation of $^{14}$C was detected in Chl *a* (Fig. 4A and B) but not in Chl *b* (data not shown). The radiosignal of Chl *a* did not significantly differ between CL and CL→HL conditions, even when the leaves were treated with Linco or NF. The $^{14}$C levels of Chl *a* was comparably high in $H_2O$-treated and NF-treated leaves throughout the chase; only the $H_2O$-treated leaves in CL showed a tendency of increasing radioactivity towards the end of the experiment. In contrast, much less radioactivity (about 40% and 50% in CL and CL→HL, respectively) was measured in the Linco-treated leaves.

Also β-C showed similarly high $^{14}C$ signals under both light conditions (Fig. 4C and D), with no or little labeling detected for xanthophylls (data not shown). The $^{14}C$ signal of β-C reached the maximal levels after 30-min chase, followed by a decrease or no substantial change until 6 h. In general, less $^{14}C$ was incorporated in β-C in the inhibitor-treated leaves compared to $H_2O$-treated leaves. Despite the treatment with NF, β-C was clearly labeled with $^{14}C$ in the Arabidopsis leaves; under CL→HL a significant reduction in the β-C radiosignal was observed with Linco but not with NF (Fig. 4D).

However, a prominent peak emerged in the radiograms of NF-treated leaves at the position of Phy (Fig. 5), the substrate of Phy desaturase, confirming the effect of our NF treatment. While only marginal levels of $^{14}C$ radioactivity were measured for Phy in $H_2O$-treated and Linco-treated leaves, strikingly high $^{14}C$ labeling of Phy was found in NF-treated leaves under CL as well as CL→HL conditions (Fig. 4E and F). The radiosignal of Phy in NF-treated leaves (>100 Net Bq µg Chl $a^{-1}$) was higher than the highest radiosignal of β-C measured in these experiments (≤80 Net Bq µg Chl $a^{-1}$). The $^{14}C$ ratio Phy:Chl *a* was about 1:2 in NF-treated leaves, with the values approaching 1:1 in CL→HL after 6 h. For comparison, the $^{14}C$ ratio β-C:Chl *a* was between 1:3 and 1:5 in $H_2O$-treated leaves, between 1:3 and 2:3 in Linco-treated leaves, and between 1:4 and 1:5 in NF-treated leaves. When the $^{14}C$ signals of β-C and Phy were added up, NF-treated leaves had 47% higher signal, and Linco-treated leaves had 49% less signal, compared to $H_2O$-treated leaves already at 0 h (Fig. 4G and H). Of the three treatments, Linco-treated leaves had the lowest total $^{14}C$ radioactivity in the sum of Chl *a*, β-C and Phy at any time point, which was about 60% lower than in $H_2O$-treated leaves.

## DISCUSSION

### Effects of Lincomycin on Pigment Turnover

The D1 protein of the PSII reaction center undergoes a continuous repair cycle in all light intensities (Tyystjärvi and Aro, 1996), going through photoinactivation, PSII disassembly, D1 degradation, insertion of a newly synthesized D1, and PSII reassembly (Baena-González and Aro, 2002). The antibiotic Linco inhibits protein synthesis in chloroplasts, thereby disrupting the D1 repair cycle and leading to accumulation of photoinactivated PSII (Tyystjärvi et al., 1992; Lee et al., 2001). This explains that the maximal PSII efficiency was lowered in Linco-treated leaves even under CL (-25% after 6 h; Fig. 1B), revealing the extent of PSII photoinactivation in leaves of CL-grown Arabidopsis plants under CL conditions. Obviously, the repair cycle could compensate this level of D1 damage in $H_2O$-treated leaves under CL (Fig. 1A). However, transfer to HL dramatically accelerated the rate of PSII photoinactivation in all three treatments (Fig. 1A), despite the operation of the xanthophyll cycle and associated photoprotective mechanisms (Fig. 3B, E and H). Under CL→HL conditions, the rate of photoinactivation exceeded the repair capacity most strongly in Linco-treated leaves (Fig. 1B).

Much less $^{14}C$ was incorporated in both Chl *a* and β-C in Linco-treated leaves compared with $H_2O$-treated leaves (Fig. 4), indicating reduced de novo synthesis of these pigments after the Linco treatment. Even though a preliminary experiment with Chl *a* fluorescence imaging manifested homogeneous inhibition of PSII in Linco-treated leaves (data not shown), the effect of this inhibitor was presumably not yet 100% during $^{14}CO_2$ application, allowing residual levels of $^{14}C$ incorporation into these pigments. The reduction of $^{14}C$ incorporation in Linco-treated leaves was more pronounced for Chl *a* than for β-C, which may indicate closer interactions between D1 protein and Chl *a* turnover. Since Chl *a* molecules and some of the Chl precursors may become harmful photosensitizers unless they are bound to proteins (Meskauskiene et al., 2001), continuous synthesis and degradation of Chl *a* may be coordinated with the D1 repair cycle. While some secondary effects of Linco cannot be ruled out (Fiekers et al., 1979), the low $^{14}C$ signal of Chl *a* measured already at 0 h (Fig. 4A and B) suggests quick downregulation of Chl *a* synthesis upon inhibition of D1 protein synthesis.

The total Chl *a* content did not change in Linco-treated leaves under CL conditions (Fig. 2D), and there was only a small, statistically non-significant decrease in Chl *a* under CL→HL at 3 h (Fig. 3D) despite serious PSII photoinhibition (Fig. 1B). Given that no more than a few % of Chl *a* molecules are bound in PSII reaction centers in leaves, changes in Chl *a* content during photoinactivation and repair is difficult to detect by measuring the total Chl *a* amount. Judging from the low but steady levels of Chl *a* radiosignal despite acute PSII photoinhibition in Linco-treated leaves after 6 h in CL→HL (Figs. 4B and 1B), both synthesis and degradation of Chl *a* seem to slow down when the D1 protein turnover is inhibited. While Chl

recycling (Vavilin and Vermaas, 2007) may maintain the steady-state $^{14}C$ level of Chl *a*, Chl *a* recycling in photoinactivated PSII is unlikely unless there is concurrent operation of D1 repair. Parallel downregulation of Chl *a* and D1 turnover suggests possible enzymatic control of Chl *a* degradation, although this does not preclude photooxidation of Chl *a* under excess light. In addition to thermal dissipation in antenna complexes, strong quenching in photoinactivated PSII (Matsubara and Chow, 2004) could reduce formation of Chl *a* triplet states and resulting production of singlet $O_2$ to minimize oxidative degradation of Chl *a*.

The effects of Linco on β-C turnover were similar to those observed for Chl *a*, albeit not as significant (Fig. 4C and D). Thus, coupling between β-C and D1 protein turnover may not be as tight as between Chl *a* and D1. This is in line with our previous finding of contrasting HL-acclimatory responses of Chl *a* and β-C turnover in Arabidopsis leaves; acclimation to HL significantly enhanced Chl *a* turnover while reducing that of β-C (Beisel et al., 2010). Unlike Chl *a*, free β-C molecules can accumulate in membranes to confer photoprotection, as can be found in leaves of field-grown plants (Verhoeven et al., 1999; Matsubara et al., 2003). Being the precursor of Z which increases strongly under excess light, as in CL→HL conditions (Fig. 3B and E), synthesis and degradation of β-C may be subject to regulatory mechanisms which are different from those of the D1 repair cycle. Contrary to the little change in Chl *a*, the marked decrease of the β-C content in Linco-treated leaves in CL→HL at 3 h (-15%) and 6 h (-30%) (Fig 3F) suggests enhanced β-C degradation, probably through photooxidation (Tracewell et al., 2001) after pronounced photoinhibition (Fig. 1B). One may ask whether β-C turnover in non-stressful conditions, like in $H_2O$-treated leaves under CL (Fig. 1A), also reflects bleaching of this pigment in PSII or is controlled by carotenoid cleavage enzymes (Auldridge et al., 2006). Hydroxylation of β-C released from photoinactivated PSII during the repair cycle, giving rise to Z increase under high-light stress as proposed for Chlamydomonas (Depka et al., 1998), is an alternative scenario to explain the observed β-C decrease concomitant with an increase in Z and V+A+Z (Fig. 3E and F) in Linco-treated leaves. However, the decreasing radiosignal of β-C in Linco-treated and $H_2O$-treated leaves in 6 h under CL→HL (Fig. 4D) did not result in $^{14}C$ labeling of Z (data not shown), suggesting that hydroxylation of β-C to Z during β-C turnover may not play a predominant role in Arabidopsis (Beisel et al., 2010).

**Effects of Norflurazon on Pigment Turnover**

The herbicide NF inhibits carotenoid biosynthesis at the step of Phy desaturation upstream of β-C. In contrast to obvious bleaching of leaves after long-term application of NF during plant cultivation (e.g. Dalla Vecchia et al., 2001), our treatment of feeding detached leaves with a 70 µM NF solution for 2 h did not entirely stop $^{14}C$ incorporation in β-C (Fig. 4C and D) and longer (overnight) treatment with NF did not enhance the inhibitory effect (data

not shown). The amount of NF taken up by leaves during these treatments was apparently not enough to saturate the cofactor (plastoquinone; Norris et al., 1995) binding site of Phy desaturase to bring about full inhibition of the enzyme (Breitenbach et al., 2001). Nevertheless, the significantly high $^{14}C$ label of Phy in NF-treated leaves (Fig. 4E and F) together with a decrease in β-C content under both CL and CL→HL conditions (Figs. 2I and 3I) demonstrates at least some effect of the inhibitor.

Inhibition of β-C synthesis by NF resulted in loss of PSII activity and D1 protein in Chlamydomonas under high light, which has been interpreted as evidence for interrelations between β-C and D1 protein turnover: photo-bleaching of β-C is thought to trigger D1 degradation while the subsequent D1 protein assembly in PSII seems to require β-C synthesis (Trebst and Depka, 1997). In our experiments with NF-treated Arabidopsis leaves under CL conditions, partial inhibition of β-C synthesis causing about 15% reduction in β-C after 3 h (Fig. 2I) had no obvious impact on Chl *a* turnover (Fig. 4A) or photoinhibition (Fig. 1C), supporting loose coupling of β-C turnover with Chl *a* and D1 turnover, as was found in the experiments with Linco. Even though the initial $^{14}C$ signal of β-C in NF-treated leaves was as high as 65% of the levels in $H_2O$-treated leaves at 0 h (Fig. 4C), it is difficult to reconcile the NF-induced significant loss of β-C (Fig. 2I) with zero PSII photoinhibition (Fig. 1C) if there is a direct causal relationship between β-C and D1 turnover. The following possibilities can be considered to explain these observations: (1) β-C is not essential for D1 repair, (2) Phy can replace β-C in PSII, (3) not all newly synthesized β-C molecules are needed for D1 repair, or (4) PSII reaction centers lacking β-C are rapidly degraded and do not accumulate, hence no reduction in Fv/Fm. Selective loss of D1 in Chlamydomonas after NF treatment (Trebst and Depka, 1997) and disappearance of PSII by deletion of Phy desaturase gene in *Synechocystis* sp. PCC 6803 (Bautista et al., 2005) rejects (1) and (2). The presence of a β-C pool not required for D1 repair and PSII activity, as assumed in (3), could explain the lack of a short-term effect of NF treatment on Chl *a* turnover and Fv/Fm under CL conditions, although 15% of the β-C pool appears too large to be dispensable for PSII activity in non-stressed leaves. As NF-treated leaves showed photoinhibition (Fig. 1C) when the decrease in β-C was more pronounced under CL→HL (Fig. 3I), rapid degradation of PSII reaction centers lacking some β-C molecules as suggested in (4) is rather unlikely.

Notably, we found ca. 50% increase of $^{14}C$ radioactivity in the sum of β-C and Phy (especially the latter) in NF-treated leaves already at 0 h (Fig. 4C–H), revealing strong and rapid upregulation of carbon flux down the carotenoid biosynthetic pathway shortly after NF treatment. Such prompt upregulation suggests a metabolic feedback mechanism in the pathway (Cazzonelli and Pogson, 2010). Activation of the Phy desaturase gene promoter, presumably via end-product regulation (β-C in this case), has been reported in tobacco seedlings grown on a NF-containing medium (Corona et al., 1996). Our data in Fig. 4 also

point to compensatory upregulation, but in the upstream of Phy, which may precede the transcriptional activation of Phy desaturase gene. The already enhanced $^{14}C$ labeling of Phy at the start of the measurement (0 h) was followed by a further increase at 6 h in the NF-treated leaves under the CL→HL conditions (Fig. 4F). This second upregulation was accompanied by a significant decrease in both β-C content (Fig. 3I) and Fv/Fm (Fig. 1C). Neither β-C decrease alone, as in NF-treated leaves in CL, nor β-C decrease combined with severe photoinhibition, as in Linco-treated leaves in CL→HL, induced a further increase in the radioactivity of Phy (or β-C) to the end of the measurement (6 h; Fig. 4C–F). In the case of β-C in Linco-treated leaves, the $^{14}C$ signal even declined. Whatever the mechanisms for the rapid (within 1 h) and slow (~6 h) upregulation of carotenoid biosynthesis in NF-treated leaves may be, some loss of β-C did occur under these conditions, suggesting that β-C degradation cannot be stopped (or downregulated) to counterbalance short supply.

The substantial loss of β-C was accompanied by a much reduced increase in V+A+Z in NF-treated leaves compared to the control under CL→HL conditions (Fig. 3H and B, respectively). Thus, inhibition of de novo carotenoid synthesis by NF can largely suppress the HL-induced increase of V+A+Z in Arabidopsis leaves, as has been observed also in duckweed (García-Plazaola et al., 2002). The residual increase in V+A+Z found in NF-treated leaves may be attributed to incomplete effects of the inhibitor in our experiment, or hydroxylation of some β-C molecules released from photoinactivated PSII during D1 repair (Depka et al., 1998). However, we were unable to detect $^{14}C$-labeled Z (data not shown), even in the $H_2O$-treated or Linco-treated samples in which Z as well as V+A+Z levels were significantly increasing under CL→HL (Fig. 3B and E). Rigorous changes in the length and timing of $^{14}CO_2$ adminstering by, for example, applying an additional second pulse at a later time or conducting a longer chase of more than 24 h did not lead to obvious $^{14}C$ labeling of Z (or other xanthophylls) in the Arabidopsis leaves. Based on these observations and considering the presumably minor role of the β-C hydroxylation during the operation of D1 repair cycle in Arabidopsis plants, it can be hypothesized that HL-induced synthesis and accumulation of V+A+Z may utilize a precursor pool which is not immediately linked to photosynthetic $CO_2$ fixation. If so, chloroplasts in mature Arabidopsis leaves may have two distinct pools and fluxes of carotenoid precursors, one for continuous synthesis of β-C in the light (rapidly deriving carbon from photosynthesis) and another for stress-induced synthesis of V+A+Z and probably also L (Fig. 3B and C). Further investigations are needed to elucidate functional and metabolic interactions between pigments and photosynthesis in green leaves.

**CONCLUSION**

Both synthesis and degradation in continuous turnover of Chl *a* seem to be regulated in coordination with D1 protein turnover in mature leaves of Arabidopsis. The β-C turnover, on the other hand, is not closely coupled with Chl *a* and D1 turnover, presumably because of photoprotective roles of β-C and downstream xanthophylls requiring a distinct control in response to stress signals. Inhibition of Phy desaturase by NF causes an initial increase in metabolic flux down the carotenoid biosynthetic pathway, as measured by a significantly higher $^{14}C$ labeling of Phy.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Arabdiopsis (*Arabidopsis thaliana*) Columbia-0 wild-type were grown in soil (ED 73 Einheitserde, Balster Einheitserdewerk) in a growth cabinet with a 12-h/12-h day/night photoperiod under constant relative air humidity of 50% and 22°C/18°C (day/night) air temperature. Photosynthetically active photon flux density of approximately 130 µmol photons $m^{-2}$ $s^{-1}$ (control light, CL) was provided by a combination of FLUORA and warm white fluorescent tubes (Osram). At the beginning and at the end of the day period, the light intensity in the growth cabinet was gradually increased or decreased over 1 h to simulate sunrise and sunset. Mature leaves (up to 3 leaves per plant) of 6- to 7-weeks-old plants were used for all experiments.

### Inhibitor and light treatments

At the end of the night period, leaves were excised from plants and the petioles immediately put in water ($H_2O$), 3 mM lincomycin (Linco) or 70 µM norflurazon (NF) solution. Leaves were let transpire for 2 h in the dark under ventilation. During the subsequent light treatment for experiments of Chl *a* fluorescence measurements, analysis of photosynthetic pigments and isotope labeling with $^{14}CO_2$, leaves were floated on water or diluted inhibitor solutions (1 mM Linco or 10 µM NF) with the adaxial surface facing the air. All experiments were conducted under CL (CL condition) or after transfer to high light (HL, 1100 µmol photons $m^{-2}$ $s^{-1}$, Master HPI-T Plus lamps; Philips) (CL→HL condition) at an ambient air temperature of 19°C.

### Chlorophyll *a* fluorescence measurements

Following different durations of exposure to CL or CL→HL conditions, the detached leaves were placed on a moist tissue and dark-adapted for 15 min by using leaf clips. Maximal PSII efficiency, Fv/Fm (fluorescence nomenclature according to van Kooten and Snel 1990), was determined by measuring Chl *a* fluorescence in dark-adapted leaves with a Handy PEA (Hansatech).

### Analysis of photosynthetic pigments

For pigment analysis leaf discs (1.54 $cm^2$) were taken from the detached leaves at different times during the CL or CL→HL treatment. The discs were frozen in liquid nitrogen and stored at -20°C for up to 2 weeks until acetone extraction. Pigment extraction and the HPLC analysis were performed according to the method described by Matsubara et al. (2005) using an Allsphere ODS-1 C18 column (5 µm, 250 x 4.6 mm; Alltech) and a corresponding guard column (5 µm, 7.5 x 4.6 mm; Alltech). Pigments were detected by a

PAD-996 UV/VIS detector (Waters) and peak areas were integrated at 440 nm with Waters Empower software. Chlorophyll content was calculated per unit of leaf area (µmol $m^{-2}$) and content of different carotenoids expressed relative to the Chl *a* amount (mmol mol Chl $a^{-1}$) of each sample.

**Isotope labeling with $^{14}CO_2$ and Radio-HPLC analysis of $^{14}C$-labeled pigments**

$^{14}CO_2$ labeling of leaves was performed in a gas-circuit system as previously described (Beisel et al., 2010). Detached leaves were supplied with water or diluted inhibitor solutions (1 mM Linco or 10 µM NF) during $^{14}CO_2$ administration released by acidification of an aqueous sodium [$^{14}C$]carbonate (1.85 MBq per leaf; GE Healthcare) under CL conditions at 19°C. Immediately after a 30-min application of $^{14}CO_2$, some leaves were harvested for analysis (time 0 h) while others were let to float on water or diluted inhibitor solutions in ambient air under CL or CL→HL conditions for different chase periods before harvest and freezing them in liquid nitrogen for radio-HPLC analysis. The radiolabeled pigments were extracted first with 1.2 mL acetone, followed by two-phase extraction (ethyl acetate and water) performed twice according to the method of Pogson et al. (1996). The extracts were concentrated to a final volume of 200 µL under nitrogen gas stream and dim laboratory light. The concentrated extracts were either immediately analyzed by radio-HPLC (100 µL injection volume) or stored at -20°C for <5 h until analysis. Radio-HPLC analysis was carried out with a Prontosil reversed-phase C30 column (3 µm, 250 x 4.6 mm; Bischoff) and a corresponding guard cartridge (3 µm, 10 x 4.0 mm; Bischoff) according to Beisel et al. (2010). The pigments were detected with a UV/VIS system (Jasco) and radioactivity by a Radioflow detector LB 509 (Berthold Technologies) with a time delay of 20 s between the two detectors (Fig. 5).
Peak integration was performed with RadioStar software (Berthold Technologies) for both UV/VIS chromatograms (286 nm and 440 nm for Phy and photosynthetic pigments, respectively) and radiograms. Peak areas of the radiogram were normalized to the Chl *a* content given by the corresponding 440-nm chromatogram and expressed as Bq µg Chl $a^{-1}$.
An extra peak found in the radiogram of NF-treated leaves (Fig. 5C) was identified as Z-phytoene (Phy) by adding pure standard of (E/Z)-phytoene (CaroteNature) to a leaf extract and measuring the absorbance at 286 nm (Fig. 5B).

**Statistical data analysis**

Pigment contents and $^{14}C$-labeling data were statistically tested by *t*-test. Pigment contents were checked for significant differences in time-course variations within each treatment by comparing data at 0.5, 3 and 6 h with 0 h. Differences in $^{14}C$ incorporation between inhibitor-treated and $H_2O$-treated leaves were tested for statistical significance at each sampling time point.

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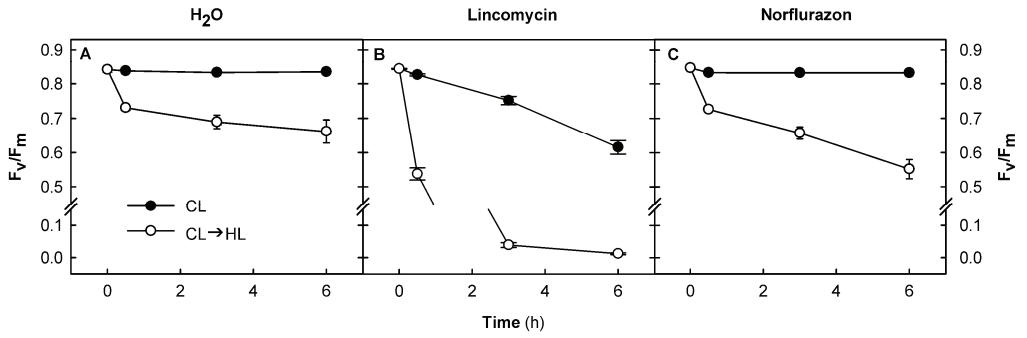


**Figure 1**

The maximal PSII efficiency (Fv/Fm) measured in leaves of Arabidopsis under different light conditions. Leaves were detached from plants grown under 130 µmol photons $m^{-2}$ $s^{-1}$ and treated with (A) water ($H_2O$), (B) 3 mM lincomycin or (C) 70 µM norflurazon for 2 h in the dark. Then, they were floated on water or diluted inhibitor solutions (1 mM lincomycin or 10 µM norflurazon), with the adaxial surface facing the air, and placed under 130 µmol photons $m^{-2}$ $s^{-1}$ (CL, black circles) or 1100 µmol photons $m^{-2}$ $s^{-1}$ (CL→HL, white circles) at 0 h. All data are means ± SE (n = 5). Error bars are shown when they are larger than the symbols.

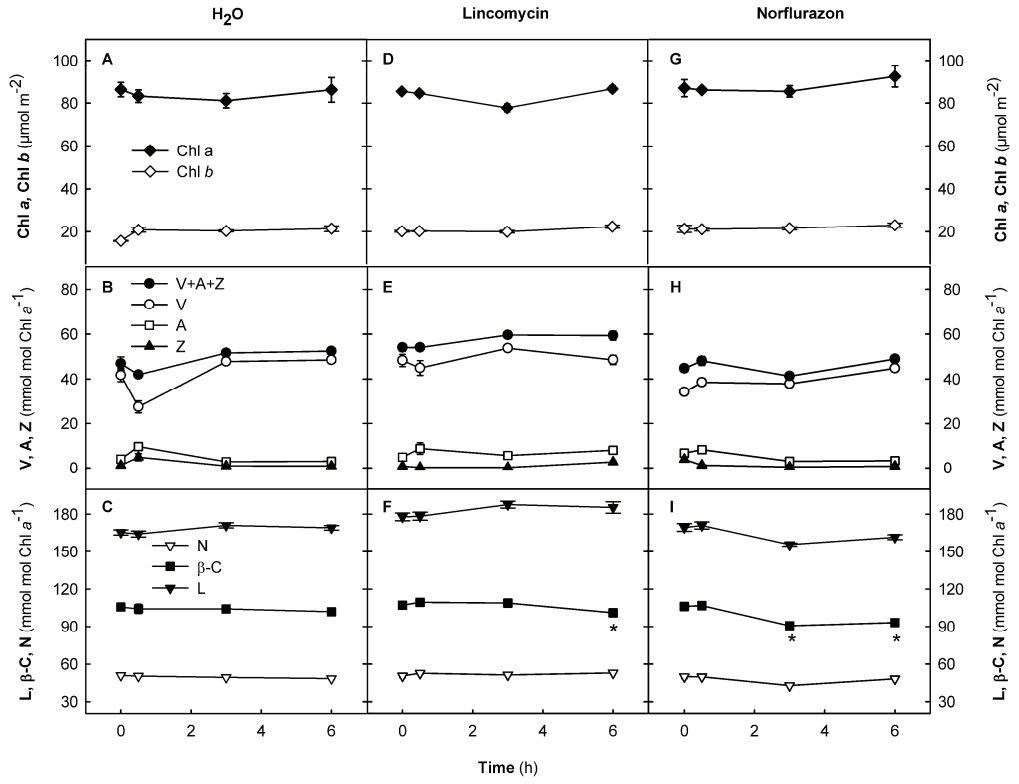


**Figure 2**

Changes in chlorophyll and carotenoid contents of Arabidopsis leaves under CL conditions. Leaves were pre-treated with (A)–(C) water ($H_2O$), (D)–(F) 3 mM lincomycin or (G)–(I) 70 µM norflurazon for 2 h in the dark prior to 0 h. Chlorophyll contents are expressed per unit of leaf area (µmol $m^{-2}$). Carotenoid contents are given relative to the Chl *a* content (mmol $mol^{-1}$ Chl *a*). Asterisks below the symbols indicate significant differences ($P < 0.01$) compared to 0 h of the same pigment for each treatment. For the xanthophyll-cycle pigments, only the data of the total V+A+Z were statistically tested. All data are means ± SE (n = 3). Error bars are shown when they are larger than the symbols.

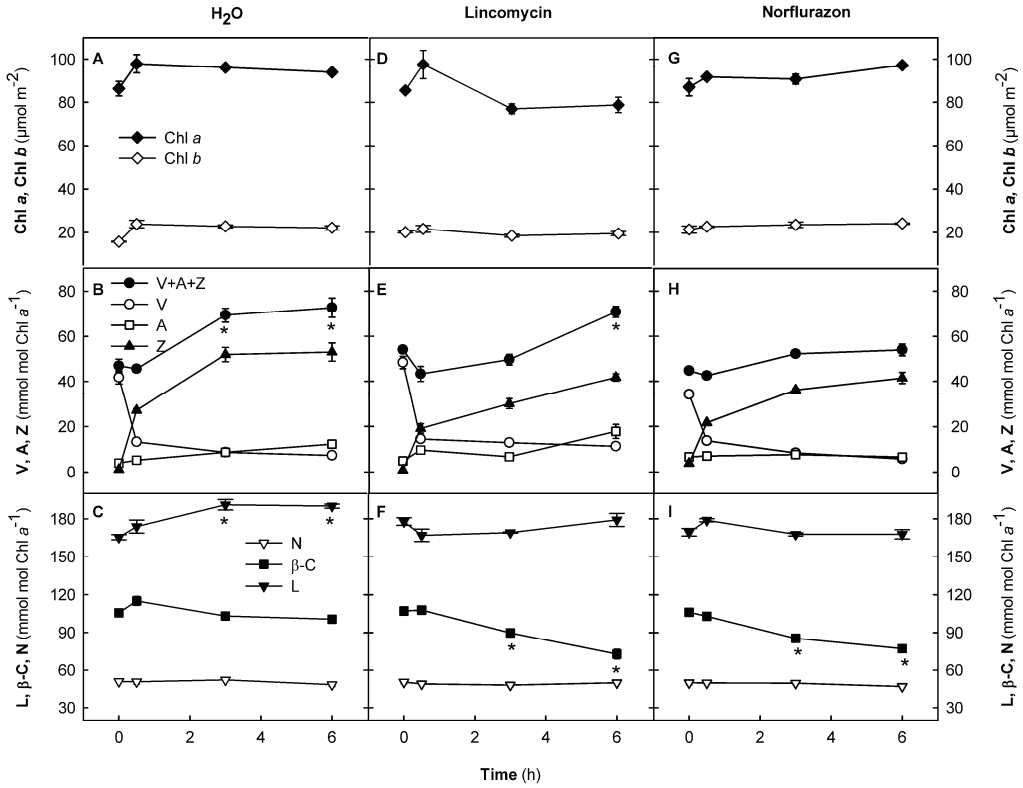


**Figure 3**

Changes in chlorophyll and carotenoid contents of Arabidopsis leaves under CL→HL conditions. Same legend as in Fig. 2.

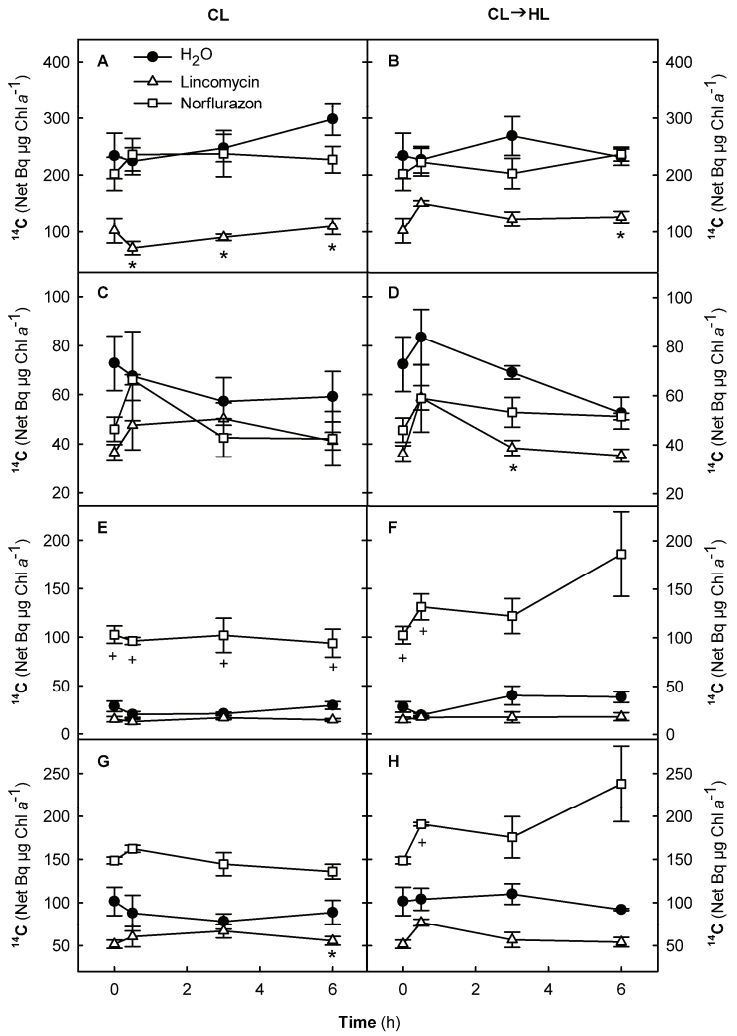


**Figure 4**

Changes in $^{14}C$ radioactivity incorporated in (A and B) chlorophyll *a*, (C and D) β-carotene, (E and F) phytoene and (G and H) sum of β-carotene and phytoene under CL or CL→HL conditions. Detaches leaves were pre-treated with water (black circles), 3 mM lincomycin (white triangles) or 70 µM norflurazon (white squares) for 2 h in the dark. Subsequently, $^{14}CO_2$ was applied to the leaves under the CL condition for 30 min (pulse period). At the end of the pulse period (at 0 h), the leaves were transferred to the ambient $CO_2$ and chase was performed under CL (A, C, E and G) or HL (B, D, F and H) for up to 6 h. The $^{14}C$ radioactivity was normalized to the Chl *a* content measured in the same samples (Bq $\mu g^{-1}$ Chl *a*). For each time point, asterisks and plus signs below the symbols indicate significant differences ($P < 0.01$) between water-treated and lincomycin-treated leaves, or between water-treated and norflurazon-treated leaves, respectively. All data are means ± SE (n = 3).

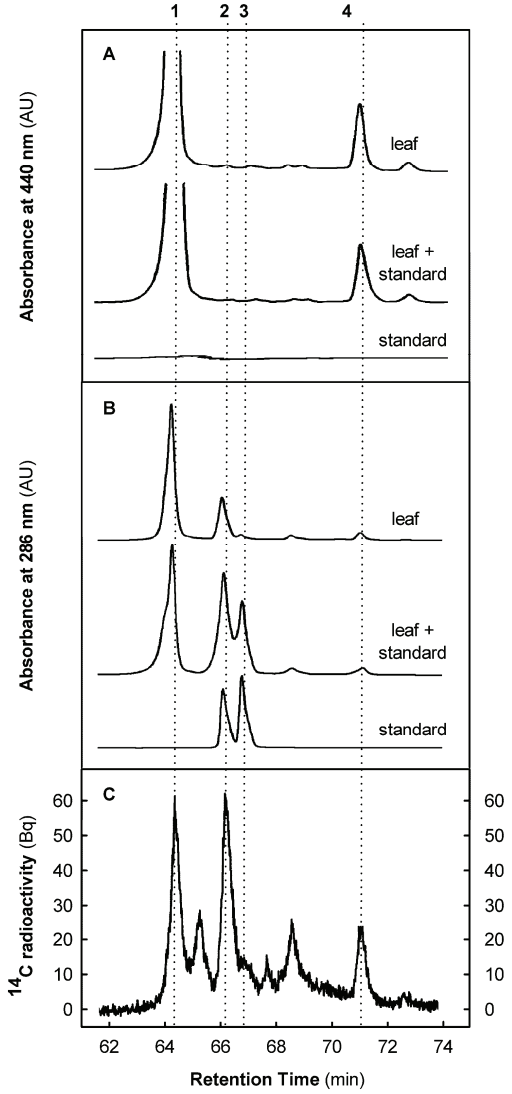


**Figure 5**

Radio-HPLC analysis for identification of $^{14}$C-phytoene. Chromatograms recorded at (A) 440 nm and (B) 286 nm. Shown are the pigment sample extracted from a norflurazon-treated leaf labeled with $^{14}CO_2$ and then exposed to HL for 6 h (leaf), the same sample after addition of 1 µg Z- and E-phytoene standard (leaf + standard), and 1 µg Z- and E-phytoene standard (standard). Peak 1, Chl *a*; peak 2, Z-phytoene; peak 3, E-phytoene; peak 4, β-carotene. AU, arbitrary units. (C) Simultaneous radiogram of $^{14}$C-labeled compounds in the leaf sample. Dotted lines indicate the expected positions of pigment peaks in the radiogram, with a 20-s offset compared with the corresponding peaks in the chromatograms due to the sequential detection by the UV-visible light and radio detectors.

# 7. List of Tables and Figures

**In this thesis**



**In Matsubara et al. (2009)**

**In Beisel et al. (2010)**



**In Beisel et al. (2010 submitted)**

# 8. List of abbreviations

| | |
|---|---|
| α-C | α-carotene |
| A | antheraxanthin |
| ABA | abscisic acid |
| ATP | adenosine triphosphate |
| AU | arbitrary units |
| β-C | β-carotene |
| Bq | becquerel (SI unit for radioactivity) |
| CCD | carotenoid cleavage dioxygenase |
| Chl | chlorophyll |
| $^{3}$Chl | triplet chlorophyll |
| CL | control light (130 µmol photons $m^{-2}$ $s^{-1}$) |
| $CO_2$ | carbon dioxide |
| $^{14}CO_2$ | carbon-14C dioxide (radioisotope) |
| CP | chlorophyll-binding protein |
| CRTISO | carotenoid isomerase |
| DOXP | 1-deoxy-D-xylulose 5-phosphate pathway |
| $e^-$ | electron |
| Fm | maximal fluorescence in the dark adapted state |
| Fo | minimal fluorescence in the dark adapted state |
| Fv | variable fluorescence (Fv=Fv/Fm) |
| GGDP | geranygerany diphosphate |
| $H^+$ | proton |
| $H_2O$ | water |
| HL | high light (1000 µmol photons $m^{-2}$ $s^{-1}$) |
| HPLC | high pressure liquid chromatography |
| L | lutein |
| LCY | lycopene cyclase |
| LHC | light harvesting complex |
| Linco | lincomycin |

| | |
|---|---|
| LMA | leaf mass per area |
| Lx | lutein epoxide |
| LYC | lycopene |
| Mg | magnesium |
| N | neoxanthin |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NF | norflurazon |
| NPQ | non-photochemical quenching |
| NXS | neoxanthin synthase |
| $O_2$ | oxygen |
| $^1O_2$ | singlet oxygen |
| OHase | hydroxylase |
| PDS | phytoene desaturase |
| Phy | phytoene |
| Phytyl-DP | phytyl diphosphate |
| PS | photosystem |
| PSY | phytoene synthase |
| qE | ΔpH-dependant thermal dissipation |
| qI | photoinhibtion |
| qT | state transitions |
| ROS | reactive oxygen species |
| SE | standard error |
| V | violaxanthin |
| VDE | violaxathin deepoxidase |
| Z | zeaxanthin |
| ZDS | ζ-carotene desaturase |
| ZE | zeaxanthin epoxidase |

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